PROTEIN DEPRIVATION AND INTESTINAL IMMUNE RESPONSES IN MICE

Alan Gordon Lamont B.Sc. (Hons.)

Thesis presented to the University of Edinburgh for the degree of Doctor of Philosophy in the Faculty of Medicine.

February 1986

SUMMARY

Using a diet containing 4% protein, I have established a model of protein deprivation in mice, and have used this to examine the effect of a single dietary deficiency on the structure and function of the immune system, with particular emphasis on the events occurring within the intestinal immune system. As a control, age-matched animals were maintained on an isocaloric diet containing 24% protein, and were subjected to identical procedures.

Protein deprived mice failed to gain weight over the first few weeks of restriction, and both coat texture and posture were altered. After a longer period on the diet, however, these mice began to gain weight steadily, although never attaining the weight of age-matched control mice. Within the small intestine, villous height and crypt depth were consistently decreased in deprived mice, but CCPR remained normal. The organization and cellularity of lymphoid tissue was markedly altered by short term protein deprivation, and this was true most notably for the thymus, followed by the spleen and lymph nodes. After longer periods of restriction, the internal organization of these organs recovered. This pattern of recovery after prolonged periods on the restricted diet was found consistently throughout this project, and was also demonstrable with the immune response to OVA.

When T cell function of deprived animals was assessed by examining help for antibody responses or DTH responses, the function was depressed after short term deprivation. However, when function was assessed by the ability to induce GvHR in F_1 animals, it was normal. Furthermore, both the antibody and the DTH response recovered after longer periods of deprivation.

Protein deprivation had separate effects on the systemic tolerance induced by feeding OVA. While tolerance for systemic antibody responses was enhanced, the degree of suppression of DTH was reduced. This effect was examined by investigating the adoptive transfer of suppression by serum or cells from OVA fed mice. Serum taken from both deprived and normal mice 1 hour after feeding a tolerizing dose of OVA transferred suppression of DTH responses, whereas spleen cells from normal, but not protein deprived mice, fed OVA 1 week previously, transferred DTH suppression to naive mice. I propose that short term protein deprivation can selectively deplete the population of T_s cells responsible for the fine control of DTH oral tolerance.

Despite the impaired oral tolerance for DTH, I was unable to induce a CMI reaction in the intestine of deprived mice after continuous oral challenge with OVA. Furthermore, there was an impaired mucosal CMI response during a GvHR in protein deprived mice. The data indicates that protein deprivation can prevent the damage to the small intestine which occurs during mucosal CMI, and I suggest that this occurs as the proliferation and migration of effector cells are inhibited within the protein deprived environment.

DECLARATION

I declare that this thesis has been composed entirely by myself, and that the work contained within it, except on occasions which are clearly stated, was performed by myself.

Acknowledgements

I am indebted to Dr Anne Ferguson for supervising this project and for her invaluable help in the preparation of this thesis. I would also like to thank Dr Ferguson's secretary, Mrs Doreen Orr, for all her help and motherly advice during my stay in Edinburgh. I am grateful to the entire staff of the Wolfson Labs. for making this a truly "tea-dependent" thesis, and particularly to Drs Maureen Bruce, Grant Munro and Richard Kay for help with animal work and discussion of results, Mrs Margaret Gordon for the ELISA and disaccharidase work, Mrs Barbara Walton for the disaccharidase work, Mrs Linda McCardle and Mr Alexander Sutherland for the histology, and Miss Janice Tocher for SEM sample preparation.

I would also like to thank Drs Allan Mowat and Stephan Strobel for helpful discussion, and the staff of the Animal Unit, Western General Hospital, in particular Miss Sharon Rossiter and Miss June Swinton, for all their help. Finally, I am indebted to the Medical Illustration Department at the Western General for preparing the figures and photographs, and I am especially grateful to Mrs Lorna Peedle for stepping in at the last moment to type the manuscript.

Many thanks to Mum and Dad for their continuing support and encouragement, and the same to Tina, Iain, Alison, Derek, Fiona, Sarah, Big Doug etc. for dispensing big gins and good cheer. A special note of thanks to the manufacturers of the "wonder drugs", Irn Bru and Solpadeine, for having made certain mornings bearable.

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ABBREVIATIONS

α	anti-
Ab	antibody
ACC	antitoxin containing cell
ADCC	antibody dependent cellular cytotoxicity
Ag	antigen
APC	Antigen presenting cell
B cell	bone marrow derived lymphocyte
BCG	Bacille Calmette-Guèrin
BCGF	B cell growth factor
BSA	Bovine serum albumin
C3	Third component of complement
C4	Fourth component of complement
CCPR	Crypt cell production rate
CFA	Complete Freund's adjuvant
Ci	Curie
CMI	Cell mediated immunity
Con A	Concanavalin A
cpm	Counts per minute
CY	Cyclophosphamide
DNA	Deoxyribonucleic acid
DTH	Delayed type hypersensitivity
e.c.	Epithelial cell
ELISA	Enzyme linked immunosorbent assay
E.M.	Electron microscopy
F_1	First generation

F_c	crystallizable fragment obtained by papain digestion of immunoglobulin
GvHR	Graft-versus-host reaction
H-2	Histocompatability locus 2 (mouse)
HSA	Human serum albumin
H + E	Haemotoxylin and eosin
Ia	Immune associated antigen
I-A	Subregion of murine H-2
i.d.	Intradermal
IEL	Intraepithelial lymphocyte
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
I-J	Subregion of murine H-2
IL-1	Interleukin 1
IL-2	Interleukin 2
i.p.	Intraperitoneal
I.U.	International unit
KLH	Keyhole limpet haemocyanin
Ly/Lyt	T lymphocyte associated antigen (mouse)
LPS	Lipopolysaccharide
M cell	Microfold cell
MDP	Muramyl dipeptide
MIF	Macrophage migration inhibition factor
MRC OX8	T lymphocyte associated antigen (rat - suppressor)
NK	Natural killer

OVA Ovalbumin

P Probability

PCV Packed cell volume

PFC Plaque forming cell

PHA Phytohaemagglutinin

PPD Purified protein derivative

PVP Polyvinyl pyrrolidone

PWM Pokeweed mitogen

RES Reticuloendothelial system

SAL Saline

s.d. Standard deviation

SEM Scanning electron microscopy

S.I. Stimulation index

sIgA Secretory immunoglobulin A

SRBC Sheep red blood cells

T cell Thymus derived lymphocyte

T_{DTH} T lymphocyte responsible for DTH

T_h Helper T lymphocyte

T_s Suppressor T lymphocyte

$T4^+$ T lymphocyte associated antigen (human - helper)

T dependent Thymus dependent

T independent Thymus independent

TRF T cell replacing factor

W3/13 T lymphocyte associated antigen (rat)

W3/25 T lymphocyte associated antigen (rat - helper)

length

cm	centimetre
mm	millimetre
μm	micrometre
nm	nanometre

volume

l	litre
ml	millilitre
μl	microlitre

weight

kg	kilogram
g	gram
mg	milligram
μg	microgram
ng	nanogram

time

hr.	hour
min.	minute

Concentration

M	molar
N	normal

Miscellaneous

pH	reciprocal $\log_{10}$ hydrogen ion concentration

symbols

<	less than
>	greater than
=	equal to
/	per

CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION TO EXPERIMENTS

Malnutrition remains a major clinical and political problem in many of the world's developing countries. The problem is not isolated here however, and it can extend to underprivileged sections of populations in industrialized nations, and even to hospital patients, where a variety of disorders may prevent consumption of adequate supplies of nutrients (Chandra,1980).

The term malnutrition encompasses both undernutrition and overnutrition, but is most commonly used to describe the syndrome where essential dietary ingredients such as protein, calories, vitamins and minerals are present in insufficient amounts. The clinical condition, marasmus, has long been recognised as resulting from an inadequate supply of energy, however it was only in the 1930s that Cicely Williams, whilst working among children in West Africa, described a condition, kwashiorkor, which resulted primarily from a deficiency of protein from the diet (Williams 1933; 1935). It became widely accepted after this that kwashiorkor was protein malnutrition accompanied by a relative excess of energy, while marasmus was under-nutrition, a lack of both protein and energy. However, from the end of the 1960s onwards, evidence has been accumulating which indicates that the difference in aetiology between kwashiorkor and marasmus was not so simple: for example, children in areas where kwashiorkor occurred

extensively, were apparently as much affected by energy deficiency as they were by a lack of protein (Alleyne, Hay, Picou, Stanfield and Whitehead, 1977). In addition to these clinical states of malnutrition, the importance of other dietary deficiencies have increasingly been recognized as forms of undernutrition. These include deficiencies in iron, zinc, vitamins, and essential fatty acids.

Despite the complexity of causes of the syndrome, it is well established that malnutrition promotes an increased susceptibility to infection, which is an important cause of morbidity in these populations (Chandra, 1980; Alleyne et al. 1977). Infection itself may contribute to the malnourished condition by inducing a loss of appetite, increased calorie requirements (e.g. through the effect of fever), and through sequestration of nutrients from metabolic pathways. If the gastrointestinal tract is infected, there is a potential risk of malabsorption and diarrhoea with loss of nutrients and tissue proteins via the stools.

The increased susceptibility and severity of infection is partly related to the adverse effect which malnutrition has on immune responses, and many investigators have examined immune function in malnourished humans and animals. These studies are almost exclusively confined to systemic immunity, and pay scant attention to the effect of malnutrition on mucosal immunity, even though, as indicated above,

gastrointestinal infection may be particularly prevalent. Evidence suggests that the mucosal and the systemic immune systems are functionally distinct (data reviewed in Bienenstock and Befus, 1980: Woloschak and Tomasi, 1983). Therefore, it is unwise to draw any conclusions on the effect of malnutrition on mucosal immunity based on the results from published work on systemic immunity. The work presented in this thesis will examine the effect of malnutrition on mucosal immunity directly.

As previously stated, malnutrition may result from deficiencies (or excesses) of a number of dietary constituents. Therefore, the inability of the malnourished host to mount an effective immune response is the net result of a number of interactions involving nutrients and immune responses. This level of complexity has prompted controlled experiments in laboratory animals focussing on a single deficiency in an attempt to answer basic physiological questions. This type of work, therefore, is not an exact parallel of clinical malnourished states. However, it can provide information about the mechanisms involved in the defective immune responses and furthermore, can allow an insight into dietary manipulation of the immune response.

As deficiency of protein in the diet can severely affect an individual's ability to survive, I decided to isolate this single deficiency in a mouse model and use this to answer questions about mucosal immunity. Based on

similar models described in the literature which were used to examine systemic immunity, diets containing 4% or 24% protein were fed to mice. These diets were isocaloric, and the low protein diet contained supplemented vitamins and minerals to attempt to avoid other dietary deficiencies.

The overall aim of the work described in this thesis was to investigate the effect of protein deprivation on the induction and expression of intestinal immunity. To fulfil this objective a number of approaches were followed:

a) The establishment of a suitable mouse model of protein malnutrition, which could be used in experiments that often required large numbers of animals, was of primary importance. The characteristics and problems involved with such a model are detailed in Chapter 4.

b) Defects in the regulation of intestinal immunity can result in the induction of a mucosal CMI response, which can be monitored through changes in gut structure (Mowat, 1981). Therefore, it was necessary to examine how protein deprivation alone altered gut structure, and the relevant results are presented in Chapter 5. These baseline studies on gut structure were extended to examine intestinal and systemic lymphoid tissue (Chapter 6) and additionally, the effect of protein deprivation on various systemic immune parameters (Chapter 7). These studies served to highlight some of the similarities and differences which existed between this model and other models of protein deprivation.

c) Both humoral and cell mediated limbs of the systemic immune response can be suppressed by feeding the antigen before its subsequent immunization (data reviewed in Bienenstock and Befus, 1980; Elson, 1985). The results of experiments which investigated the ability of protein deprivation to affect this orally induced tolerance are detailed in Chapter 8. Further investigations into the mechanisms involved, using serum and cell transfer protocols are presented in Chapter 9.

d) Finally, the experiments in Chapter 10 examine the consequences for the gut of a disturbance in the normal regulation of mucosal immune responses arising from short term protein deprivation.

The remainder of this chapter will contain a review of the literature concerning the effects of protein deprivation both on immune responses and on the structure and function of the small intestine. Most of the data will pertain to animal models of protein deprivation. However, where relevant, data from observations on malnourished human populations will be included. The next chapter will briefly review the large body of existing knowledge on the phenomenon of oral tolerance.

THE EFFECT OF PROTEIN DEPRIVATION ON IMMUNE RESPONSES

This section will concentrate on those reports which contribute significantly to our understanding of the problem in general. For more detailed reviews, the reader is referred to two excellent books by Suskind (1977) and Chandra (1980), and reviews by Scrimshaw, Taylor and Gordon (1968), Gross and Newberne (1980), and Keusch (1981).

Histopathology

Dietary protein deprivation induces a generalized loss of lymphoid tissue which is greater than the loss of total body weight (Bell, Hazell and Price, 1976(a)). This severity of effect is due mainly to the high rate of protein turnover which occurs in these lymphoid organs (Deo and Mathur, 1975). The organ which appears to be most affected is the thymus, and this is followed, in severity of effect, by the spleen and mesenteric lymph nodes (Bhuyan and Ramalingaswami, 1974; Bell *et al*. 1976(a)).

After short term protein deprivation, pronounced thymic atrophy is observed, and this is accompanied by a low cell yield from this organ (Aschkenasy, 1975; Bell *et al*. 1976(a)). The weight loss is primarily due to a reduction in size of the thymic cortex, with the medulla being largely unaffected. The junction between cortex and medulla is also less pronounced (Bell *et al*, 1976(a)).

The cell and weight loss of the spleen in protein deprived mice is not as extreme as in the thymus. Extra

medullary haemopoiesis and myelopoiesis is severely curtailed and consequently, there is a considerable reduction in red pulp area (Bell, Hazell and Sheridan, 1976(b)). Lymphoid follicles in the white pulp are reduced in size, although germinal centres are still present. The marginal sinus areas lose their definition and, as a result, demarcation between the red and white pulp is less apparent (Bell et al. 1976(a)). The loss of cells is greatest in the T-dependent areas of the spleen, particularly through depletion of non-migratory T cells, whereas recirculating T cells and resident B cells appear to be least affected by nutritional insult (Aschkenasy, 1975; Bell et al. 1976(a)). The latter authors also remark that the severe pathological changes associated with protein deprivation are not observed in every animal, although most do show them to some extent, and they suggest therefore, that some animals show better adaptation to the diets than others. This feature of differential adaptation to a low protein diet in this kind of work is a common one, and is one source of variation between different models of protein deprivation.

The mesenteric lymph nodes are least affected by protein deprivation. Although the organ is reduced in size, the histological appearance is normal, with well-maintained germinal centres and cortical areas (Bell et al. 1976(a)). Lymphoid cells in the germinal centres show decreased mitotic rate with an increase in cell turnover time (Bhuyan and Ramalingaswami, 1974), and these authors suggest that

few cells are entering mitosis after the DNA synthesis phase. Additionally, it is possible that protein deficiency can prolong the DNA synthesis phase as has been reported from observations of regenerating livers from protein deficient rats (Deo, Mathur and Ramalingaswami, 1967). The relative resistance of mesenteric lymph nodes compared to the thymus in protein deprivation is interpreted to be a consequence of the stimulation by gut derived antigen which these nodes receive (Bhuyan and Ramalingaswami, 1974; Bell et al. 1976(a)).

Very little information on the effect of protein deprivation on GALT structure is available. Aggregates of lymphoid cells in the small intestine of malnourished children are reduced in size (Chandra 1979(a)), however Bell et al. (1976(a)) have reported that Peyer's patches of protein deprived mice retain their size and structure. Lymphocytes occurring within the villus epithelium (intraepithelial lymphocytes; IEL) form an integral part of the GALT, and a number of studies have examined their frequency in protein deprived intestine. In young rats deprived of protein, the relative and absolute number of IELs is reduced, the findings being similar to those where the rats are neonatally thymectomised (Chandra, 1979(b)). Protein deficiency in adult rats also results in a reduction in jejunal IELs. Moreover, the proportion of lymphocytes crossing the basement membrane of the epithelium is increased, and this is interpreted to suggest that the decrease in IEL count is the result of these cells leaving

the villi via dilated lymphatics in the lamina propria (Maffei, Rodrigues, Camergo and Campana, 1980). Using monoclonal antibodies specific for markers on rat T lymphocytes, Lyscom and Brueton (1983) discovered that post weaning protein malnutrition depresses IEL numbers and delays the development of MRC $OX8^{+}$, $W3/13^{-}$ cells. This subset of IELs represents a large proportion of the total number of IELs, but is of unknown function (Lyscom and Brueton, 1982).

It is clear that two major physiological processes underlie the loss of weight in lymphoid tissue. First and most obviously, cell division and proliferation are severely restricted in protein deprived animals. Secondly, the stress induced by protein deficiency can result in increased levels of corticosteroid hormone in the plasma of rats (Aschkenasy, Adams and Joly, 1966). One major target of corticosteroids is thymolymphatic tissue and the lympholytic and immunosuppressive qualities of steroids are well known. Experimentally, Bell et al. (1976(a)) have shown that the profound involution of lymphoid tissues in protein deficient mice is largely prevented by adrenalectomy.

Humoral Immunity

1. Systemic antibody responses

A large amount of research with animal models of protein deprivation has focussed on the antibody response. These

models have provided scientists with much information, but the variation which exists between models often results in widely differing conclusions being drawn. This variation stems from factors such as the length and severity of the restriction, species and strain of the animal model, and perhaps most importantly, from the type of antigen used.

The most commonly employed antigen in these types of experiments is sheep red blood cells (SRBC) and many reports have shown that protein deprivation can decrease the splenic plaque forming cell (PFC) response to this antigen (Kenney, Roderuck, Arnrich and Piedad, 1968; Mathur, Ramalingaswami and Deo, 1972; McFarlane and Hamid, 1973; Cooper, Good and Mariani, 1974). The decrease in PFC number correlates with a reduction in serum antibody titre, and it is suggested that the production of antibody plasma cell is normal with the reduced titres resulting solely from a decrease in plasma cell number (Kenney et al, 1968; Cooper et al, 1974). Aschkenasy (1973) has also reported that serum antibody titres to SRBC are depressed in protein deficient animals although rosette forming cells (RFC) are found in numbers equivalent to control levels. In this work, he has shown that cellular proliferation after antigenic exposure is greatest in B cell areas and least in T cell areas, and he suggests that the relative increase in RFC observed is due to a reduction in T cell numbers. This selective effect of protein deficiency on the thymus dependent limb of immunity is also suggested by the finding that a normal response to

SRBC in protein deficient mice can be restored by injection of normal syngeneic thymocytes (Mathur et al, 1972).

The effect of protein deprivation on the serum antibody response to SRBC is also dependent on the dose of antigen received. At high doses, antibody responses are markedly reduced, but at low doses, both primary and secondary responses are near normal (Price and Bell, 1977(a)). These authors also noticed that proportions of IgM produced during the course of the response is increased. Finally, although the majority of reports show a decreased antibody response to SRBC, one study indicates that even after dietary protein has been lowered to a level of 4%, total IgM and IgG responses to SRBC are similar to those from control animals (Malavé, Pocino and Baute, 1983).

The response of protein deprived animals to other types of T-dependent antigen have also been extensively studied. Price and Bell (1977(b)) have found in their studies with tetanus toxoid a similar situation to that with SRBC. Low doses of toxoid result in normal antibody responses, but with high doses, the response of protein deficient mice is generally decreased. Again, a greater proportion of IgM is present in the responses of these mice. Malavé and Layrisse (1976) evaluated the effect of protein deficiency in C57BL/6J mice on the antibody response to alloantigens of DBA/2 mice. In protein deficient mice, the number of IgM secreting cells per 10^7 spleen cells is increased to twice the control level, although serum levels of IgM antibody are only slightly increased in the primary response.

In the secondary response, the serum IgM antibody is markedly depressed. IgG production to alloantigenic determinants is also decreased in both the primary and secondary response.

It has previously been reported that levels of IgM anti-H-2 antibodies are not depressed after neonatal thymectomy, while the IgG response to H-2 antigens is T-dependent (Klein, Livnat, Hauptfeld, Jerabek and Weissman, 1974). Therefore, the marked inhibition of IgG responses in protein deprived mice in the above studies could indicate that protein restriction affects either a T cell population with helper activity in the T-dependent antibody response or a T-dependent B cell subset (Malavé and Layrisse, 1976). Alternatively, the poor IgG response to a variety of antigens in protein deprived mice may result from selective impairment of cells governing the IgM → IgG switch (Malavé and Layrisse, 1976).

By using T-independent antigens to study humoral immune function, it is observed that B cell function is normal or even enhanced by protein deficiency. Antibody responses to B.abortus are slightly decreased at high antigen doses but are normal or elevated at low antigen doses with greater proportions of IgM present (Price and Bell 1977(a)). The same author has also reported that the primary PFC response to polyvinyl pyrrolidone (PVP) is enhanced in protein deprived mice (Price,1978).

The ability of protein deprivation to impair the antibody response to T-dependent antigens has important implications for CMI responses to tumour cells. Specific serum factors which block cellular immunity *in vitro* and enhance specific tumour growth *in vivo* reside in the 7S gamma globulin fraction of serum (Bruner, Mauel, Cerrottini, and Chapius, 1968) and further work has identified an IgG_2 molecule as being important in mice, in this respect (Takasugi and Hildemann, 1969). The more rapid rejection of skin allografts in protein deficient mice (Cooper, Mariani and Good, 1974) may be due to a failure of these animals to develop a specific antibody which blocks the anti-graft response, and evidence for this has been obtained using a tumour heterograft system in protein deficient rats (Jose and Good, 1971).

Despite the importance of nutritional supplementation as a means of improving the efficiency of vaccination programmes in malnourished populations (Chandra, 1980), only one study has examined the effect of nutritional rehabilitation on humoral immunity in protein deprived animals. The experiments in this report show that nutritional rehabilitation begun up to five days prior to immunization with SRBC, tetanus toxoid, or *B.abortus* results in profound suppression of antibody production to these three antigens (Price and Bell, 1976).

However, transfer to the high protein diet on the day of immunization, or feeding deficient mice the normal diet for only 2 days at the time of injection, produces higher titres than does transfer a few days before immunization. It is surprising that this report has not been extended in later studies, as it reveals the potential complexity of events which surrounds the recovery of the immune response after nutritional rehabilitation. These findings argue for caution in the design of immunization procedures for malnourished populations.

While studies of humoral immunity in vivo have proved useful, it was recognised that the proliferation of antigen sensitive lymphoid cells may be particularly affected in the restrictive environment. Therefore, although some events in the antibody response to T-dependent antigens may be normal, e.g. antigen presentation to T_h cells, and T cell help for B cells, proliferation of both T_h cells and B cells may be impaired, thus giving the phenotype of a depressed response. To eliminate this problem, Pocino and Malavé (1981) have studied the influence of protein restriction on the antibody response of spleen cells which have been stimulated in vitro with antigen. Short or long term protein restriction, initiated after weaning, leads to increased PFC responses to SRBC, TNP-Ficoll and TNP-LPS. Furthermore, experiments using mixed cultures of antigen sensitized

lymphocytes with fresh non-immune cells from either protein deprived and normal donor mice were performed, and the result suggests that protein deficiency selectively depletes short lived suppressor effector lymphocytes which are activated in the presence of antigen stimulated inducer cells.

Finally, humoral immunity depends not only on the quantity of antibody produced in response to antigen, but also on its affinity for antigen. Passwell, Steward and Soothill (1974) demonstrated that protein deficient Ajax mice produce antibody of lower affinity for antigen than control mice. These findings were later extended to show that this effect of protein deprivation is important only in mice normally producing high affinity antibody, and does not happen with low affinity antibody producers (Reinhardt and Steward, 1979). Therefore, antibody levels alone may not accurately reflect humoral immune function.

2. Local antibody responses

In contrast to the volume of literature available about systemic antibody responses, few reports concentrate on the effect of protein deprivation on local antibody responses at the level of the intestinal mucosa. These reports are mainly confined to observations of secretory IgA levels in malnourished human populations, although some information from protein deprived animal models is available.

Nasal secretions from malnourished children have been found to contain low levels of secretory IgA (Sirinisha, Suskind, Edelman, Asvapaka and Olson, 1975). The secretory IgA antibody response following immunization with measles virus vaccine is reduced in malnourished children compared to healthy controls (Chandra, 1975(a)). Further to this, Chandra has also observed that malnourished children have fewer IgA plasma cells in their jejunal mucosa than normal controls (Chandra 1979(a)). An interesting point to note with this work is that the jejunum of these children contains increased numbers of both IgM and IgG plasma cells.

Because of the obvious difficulties involved, levels of intestinal secretory IgA in malnourished populations have not been determined. Therefore, direct correlations between the impaired local antibody response and the increased bacterial contamination of the small intestine of malnourished children remain speculative at the moment. Despite this fact, the decrease in secretory immunity has been implicated as the major factor in the increased frequency of gastrointestinal infections (Sirinisha, Suskind, Edelman, Asvapaka and Olson, 1977).

The development of gut IgA plasma cells has been examined in a mouse model of protein deprivation (Wade, Lemonnier, Alexiu and Bocquet, 1982). These authors have shown that although a limited period of deprivation has no effect on the development of IgA plasma cells, a more

severe and prolonged period of malnutrition does decrease their number. The excellent study of Barry and Pierce (1979) reports that protein deficiency markedly impairs the mucosal immune response to cholera toxin as measured by numbers of anti-toxin containing cells (ACC) within the intestinal mucosa. The reasons forwarded to explain this are a decrease in the generation of specific IgA immunoblasts within the Peyer's patches, and an impairment in antigen driven division of immunoblasts within the mucosa. Alternatively, it could also be explained by a defect in localization of the blast cells within the intestinal mucosa, as suggested by the work of McDermott and his colleagues (McDermott, Mark, Befus, Baliga, Suskind and Bienenstock, 1982).

Finally, secretory IgA from gut washings of protein deprived mice has been measured (Lim, Messiha and Watson, 1980). These authors demonstrated that after 6 weeks protein deprivation, levels of IgA in gut washings are equivalent to those from control mice, but significantly decreased following prolonged periods of restriction.

Cell Mediated Immunity

Although this section is intended to review the literature describing the effect of protein deprivation on T cell number and function, I shall also include information on natural killer (NK) cell and antibody dependent cellular cytotoxic (ADCC) activity. As mentioned previously,

cellular proliferation may be impaired within a malnourished host. Therefore *in vivo* tests of CMI might reveal a defect in function caused by protein deprivation which *in vitro* tests would not. For this reason, I shall deal with these types of tests separately.

1. T cell number

Numerous reports have been published which show that protein malnutrition decreases absolute numbers of T lymphocytes in both animal models and human populations (Aschkenasy, 1973; Jose, Stutman and Good, 1973; Chandra, 1974; 1983). Moreover, different subsets within the T cell population are relatively more affected than others. For example, in malnourished children, the proportion of lymphocytes with receptors for the F_c portion of IgM (T_μ: "helper" cells) are decreased, and those with receptors for the F_c portion of IgG (T_γ: "suppressor" cells) are slightly increased. There is also a relative increase in the proportion of null cells, which do not bear conventional T or B lymphocyte markers (Chandra, 1979(c)). The same author has also reported a decrease in both number of $T4^+$ cells, and in their ability to provide help for B cells (Chandra, 1983).

In mice, one study has examined the disparate effect of protein deprivation on different subsets of T cells based on the old classification of T_1 cells (short-lived, sessile T lymphocytes), and T_2 cells (long-lived, recirculating T lymphocytes: Cantor and Boyse, 1977). During the first

weeks of dietary restriction selective depletion of non-recirculating T_1 cells leads to a relative enrichment of recirculating T_2 cells (Malavé, Nemeth and Pocino, 1980). From this observation, the authors suggest that the increased response of protein deprived lymphocytes following stimulation with high doses of mitogen in their experiments, may reflect decreased function of suppressor cells within the T_1 population.

2. In vivo tests for CMI

One of the most widely used tests to examine CMI function in vivo, is the delayed type hypersensitivity (DTH) reaction. Different workers have used the antigen SRBC to examine the effect of protein deprivation on the DTH response, and have found impaired responses in animals maintained on 4% protein diets for short periods of time (Narayanan, Nath, Bhuyan and Talwar, 1977; Malavé, Pocino and Baute, 1983). When protein deprivation is induced using a diet containing 8% protein, the DTH response to SRBC is not impaired. Furthermore, while immunization with large numbers of SRBC suppresses the DTH response in normal, protein-sufficient mice, this fails to suppress the response of protein deprived mice, a fact which the authors attribute to a lack of suppressor cells in these mice (Malavé et al. 1983). The adverse effect which protein deprivation has on DTH responses to purified protein derivative (PPD) of tuberculin can be reversed by refeeding with a normal protein sufficient diet

for two weeks prior to challenge (Sakamoto, Nishioka and Shimada, 1979).

As the DTH response broadly consists of an afferent limb, an efferent limb, and an inflammatory limb (see Fig. 7.4), a defect in any of these components may account for an impaired skin test response (Edelman, 1977). Therefore, interpretation of poor DTH responses in protein deprived animals, in terms of mechanisms, is difficult. Even if T cell function is maintained, incompetent leucocyte infiltration into the site of antigen exposure, as is observed in protein deprived rats (Gray, 1964), will prevent a response.

Bhuyan and Ramalingaswami (1973) studied the early inductive phase of the cellular immune response by evaluating the development of tubercules in draining lymph nodes of protein deprived guinea pigs after intradermal immunization with BCG. They found a marked delay and defective mobilization of macrophages, which results in a poorly formed primary BCG nodule. The draining lymph nodes are atrophic, with no evidence of cellular proliferation in the paracortical areas. The authors proposed that protein deficiency can restrict both macrophage and T cell function in this model.

The use of allogeneic graft rejection as a measure of CMI function in protein deprived mice has suggested a different conclusion. Protein deprived rats reject skin grafts at a rate comparable to that of controls (McFarlane and Hamid, 1973), while some workers have demonstrated

accelerated graft rejection in protein deprived mice (Jose, and Good, 1971; Cooper et al. 1974). The enhanced rejection is not related to the degree of vascularization of the grafted skin, however, the absence of blocking antibody to the graft may be important (Jose and Good, 1971).

Of the various reactions mediated by the cellular limb of the immune response, the GvHR is very useful as a means of examining the immunocompetence of the donor T cells. Several workers have used this test to study the function of protein deprived parental cells in a normally nourished F_1 environment, in this way removing the restricting influence of protein deprivation on cellular proliferation and migration. Cooper et al. (1974) showed that spleen cells from protein deficient donors induce a significantly greater splenomegaly in F_1 recipients than do normal cells. This finding was confirmed by Bell and Hazell (1975), and extended to show that thymus, Peyer's patch, and mesenteric lymph node cells from protein deprived parental donors also have increased reactivity in the GvHR assay. The authors attribute this phenomenon to the persistence of long-lived GvHR inducing T cells in protein deprivation, while short-lived cell populations are selectively depleted. One report has examined the ability of normal parental spleen cells to induce a local GvHR in the regional lymph node of a protein deprived animal and has found that the reaction is depressed (Aschkenasy, 1976).

Adoptive transfer of cells from syngeneic protein deprived donors to protein sufficient recipients has been used to examine the effect of reduced protein intake on cells which can regulate systemic and local antibody responses. By a combination of cell transfer experiments from normal and protein deficient donors into recipients of both dietary groups at the time of immunization, Price and Turner (1979) suggest that responses to PVP in mice are determined by distinct "suppressor-inducing" and "suppressor" cells which act via helper T cells. The ratio of helper to suppressor cells in protein deficient mice appears to be increased, a fact which may contribute to the increased responses to PVP in these mice (Price, 1978). The cells which regulate the mucosal antibody response to cholera toxin are also affected by protein deprivation (Koster and Pierce, 1985). These studies confirm the earlier finding of decreased mucosal IgA responses in protein deprived rats (Barry and Pierce, 1979), and show that the generation of cells which can transfer priming or suppression of this response to naive recipients, is impaired.

In summary, the various in vivo tests used to examine CMI function in protein deprivation provide conflicting results; while tests for DTH responsiveness suggest impaired function, the results from graft rejection and GvHR studies indicate normal or enhanced function.

Of the latter types of studies, graft rejection is probably the hardest to interpret because of the absence

of enhancing antibody in protein deprived animals. The selective effect of protein deficiency on short-lived suppressor cells is the simplest way to explain increased GvHR activity, and this conclusion is borne out by the cell transfer studies. By removing lymphoid cells from the malnourished environment, the potential for these cells to migrate and proliferate may be restored. Therefore, it appears likely that the decreased DTH response may be due largely to the impaired migration of both specific and non-specific effector cells to the site of antigen challenge. A direct effect of protein deprivation on the T_{DTH} cell itself, however, cannot be excluded.

3. In vitro tests for CMI

Transformation of lymphocytes in vitro following exposure to mitogens has provided scientists with a test to measure lymphocyte reactivity. However, while there is general agreement that lymphocyte transformation to PHA in malnourished humans is decreased (Chandra, 1980), results from animal models prove conflicting. McFarlane and Hamid (1973) compared the responses of malnourished and control rats to PHA (T cell mitogen) and PWM (B cell mitogen), and found that control rats respond better to PHA than PWM, but the situation is reversed in malnourished rats. Alternatively, results from two studies in protein deprived mice indicate that PHA transformation is increased over that of control mice (Cooper et al., 1974; Malavé et al.,

1980). The reasons for this disparity may be sought in the different regimes of inducing protein deprivation between models, but may also result from the different methods used to express the results of the transformation tests.

Despite the importance of lymphokines as immune response signals, very little information is available concerning the effect of protein deprivation on lymphokine production. Kramer and Good (1975) reported that protein-deficient guinea pigs immunized with BCG produce equivalent amounts of migration inhibition factor (MIF) compared with controls even at a dietary protein level of 3%. In addition, guinea pigs on 6% dietary protein are capable of producing the factor in response to doses of antigen lower than those capable of eliciting a response in normal animals. IL-2 production in protein deprived animals in response to Con A stimulation has also been shown to be equivalent to levels produced from control mice (Saxena, Saxena and Adler, 1984).

The majority of studies concerning CMI function in protein deprived animal models have examined principally T cell mediated phenomena. However, also of major importance in cellular cytotoxicity is NK and ADCC activity, and recently a few reports have focussed on these limbs of the CMI response. Lymphoid cells, isolated from the intestinal mucosae of protein deprived mice were examined for their ADCC activity against antibody coated SRBC *in vitro* (Lim *et al.*,1981). Although activity increases with time on

the diet, this is always less than in those animals which had been maintained on a protein sufficient diet for the duration of the experiment. Splenic NK activity in protein deprived mice is initially increased over control levels, but after 7 weeks, this returns to normal or subnormal values (Saxena et al.,1984). In contrast, measurements of NK activity in the peripheral blood lymphocytes of children with marasmus or kwashiorkor are consistently decreased (Salimonu, Ojo-Amaize, Johnson, Laditan, Akinwolere and Wigzell, 1983).

Phagocytic cell function

Although cells of the reticuloendothelial system play an important role in the immune system, very few studies have investigated the effect of protein deprivation on their function. Most of these studies are limited to an examination of phagocytosis and intracellular killing, while only one report is concerned with the vital role of accessory cells in processing and presentation of antigen to lymphocytes.

Clearance of PVP from the circulation after intravenous injection is impaired in protein deprived mice (Coovadia and Soothill, 1976; Reinhardt and Steward, 1979), and this same effect occurs when colloidal carbon is used (Passwell, Steward and Soothill, 1974). The decreased rate of clearance results solely from the reduction in numbers of reticuloendothelial cells, with the function of the remaining phagocytic cells being increased (Coovadia

and Soothill, 1976). Evidence supporting this statement is contained in two reports which show enhanced phagocytosis of antigen by macrophages from both protein deprived mice and rats (Cooper et al.,1974; Moriguchi, Sone, Tachibana and Kishino, 1983). Macrophage bactericidal activity studied in protein deprived animals, is normal (Bhuyan, Mohapatra and Ramalingaswami, 1974; Keusch, Douglas, Hammer and Braden, 1978).

The immunogenic function of macrophages has recently been investigated in protein deprived mice (Rose, Holt and Turner, 1982). Antigen pulsed macrophages from protein deprived mice can prime naive recipient mice for IgE and IgG responses as efficiently as those from normal mice. Uptake and breakdown of radiolabelled antigen is comparable with normal mice, however, the antigen pulsed macrophages from malnourished mice are deficient in their capacity to trigger proliferation of antigen primed T cells in vitro. This is the only report to date which suggests that impaired antigen presentation in malnourished populations may contribute to the defect in immune responses.

Complement and other non-specific factors

The most complete study of individual complement component levels in malnourished humans is that of Sirinisha and his colleagues (Sirinisha, Edelman, Suskind, Charupatana and Olson, 1973). They found significant depression in the

levels of every component except C4. C3 seems to be the most affected. In a rat model of protein deprivation, levels of serum C3 are also particularly depressed (McGhee, Michalek, Ghanta and Stewart, 1974). It is thought that decreased protein synthesis in the liver and increased consumption of complement components may underlie changes in the complement system.

Lysozyme concentration has been measured in plasma and neutrophils from malnourished human populations, and the results demonstrate decreased levels compared to well nourished controls (Chandra, Khahil, Howse, Chandra and Kutty, 1977). Lysozyme concentration is also reduced in tears (Watson, Reyes and McMurray, 1978). Finally, serum transferrin concentrations are low in persons with kwashiorkor, and they have been shown to correlate inversely with mortality (Antia, McFarlane and Soothill, 1968).

THE EFFECT OF PROTEIN DEPRIVATION ON SMALL INTESTINAL STRUCTURE AND ABSORPTIVE FUNCTION

This section contains information from both animal models of protein deprivation and from clinical observations of malnourished human populations. Because of variability in degrees of malnutrition, e.g. marasmus versus kwashiorkor, the spectrum of gastrointestinal dysfunction is considerable. There is wide variation also between the results from experimental animal studies, mainly due to the different methods for inducing malnutrition. Therefore, interpretation

of these results is difficult, and the conclusions confusing. In general, however, morphologic and functional changes occur even after short periods of protein deprivation.

Morphologic changes

Jejunal biopsies from malnourished infants reveal that the intestine of marasmic children is much less severely affected than that of children with kwashiorkor (Walker, 1980). The mucosa of marasmic children is similar to normal controls although much thinner. In contrast, the mucosa of kwashiorkor children takes on an appearance similar to that observed with coeliac disease, with subtotal villous atrophy and a flattening of the top of the mucosa (Brunser, Reid, Monckeberg, Maccioni and Contreras, 1966). Additionally, other studies have observed that the crypt depth is increased, with the lamina propria infiltrated with inflammatory cells (Stanfield, Hutt and Tunnicliffe, 1965; Tandon, Mogatra, Saraya and Ramalingaswami, 1968).

Protein deprivation has been shown to decrease both villous height and cell turnover in rats and monkeys (Hopper, Rose and Wannemacher, 1972; Deo and Ramalingaswami, 1965). Crypt depth is reduced compared to normally fed controls, and the rate of migration of epithelial cells from the base to the tip of the villus is also decreased (Guiraldes and Hamilton, 1981). Cellular proliferation in the crypts of protein deprived rats is reduced, and it is stated that the DNA synthesis phase and the mitotic phase of the cell

cycle are the most affected by this nutritional insult (Hopper, Wannemacher and McGovern, 1968).

One report has examined the effect of protein deficiency on the pattern of maturation of the gastrointestinal tract after weaning (Syme, 1982). This effect is not consistent: for example, although the villous height of protein deprived rats is reduced 28 days after weaning compared to normally fed, age-matched controls, it is normal when measured 7 and 70 days after weaning. It is suggested that protein deficiency can delay maturation of the small intestine by either slowing or inhibiting changes seen in normal maturation. This effect is more pronounced with greater degrees of deprivation.

Functional changes

Several important gastrointestinal functions are altered in patients with malnutrition. Barbezat and Hansen (1968) have observed that both resting and stimulated pancreatic enzyme levels, including lipase, trypsin, chymotrypsin and amylase, are decreased in malnourished conditions. This impairment in pancreatic functions alters intraluminal digestion, and may further contribute to the maldigestion and malabsorption problems which accompany malnutrition (Walker, 1980). Impaired pancreatic function has also been reported in a protein deprived rat model (Hatch, Lebenthal, Krasner and Branski, 1979).

Brush border digestive enzyme activity develops as the epithelial cell migrates up the villus, and this has been extensively studied in protein malnutrition. Total small intestinal activity of lactase, sucrase, alkaline phosphatase and thymidine kinase is lowered in protein deprived rats, however, when the activity is expressed per gram of mucosal protein, lactase activity is increased (Guiraldes and Hamilton, 1981). Increased lactase activity with diminished sucrase and maltase activity is found in another report (Hatch et al.,1979), and the authors have suggested that the pattern of activity of a malnourished rat resembles that of an immature normal rat. In contrast, disaccharidase activity in two rat models of protein deprivation is normal compared to control rats (Madi, Jervis, Anderson and Zimmerman, 1970; Prosper, Murray and Kern, 1968).

The development of brush border peptidase and disacharidase activity of individual enterocytes in normal and protein deprived intestine has been examined using microdensitometry techniques. While peak activity of aminopeptidase N is lower in low protein enterocytes compared to normally fed enterocytes, this situation is reversed when isomaltase activity is examined. In addition, protein deprivation does not alter the position on the villus where enzyme activities are maximal (King, Paterson, Peacock, Smith and Syme, 1983).

Intestinal flora play an important role in non-specific host defence, and alterations in composition may render the

host more susceptible to pathogenic organisms. Studies from malnourished human populations suggest a change in the concentration, type, and distribution of normal flora. For example, malnourished children with weanling diarrhoea and adults with malnutrition are found to have colonies of anaerobes in the stomach, duodenum, and jejunum, which well-nourished controls do not have (Neumann, 1977). A reduction in gastrointestinal mucus production is indicated by the decreased number and size of goblet cells in protein deprived rat mucosa (Madi et al., 1970).

The important intestinal function of absorption of nutrients has been examined in protein deprived animals using different experimental designs. First, uptake of the amino acid, valine, at the level of the enterocyte has been examined in vitro (Syme and Smith, 1982). It is only the older cells near the tip of the villus which can absorb valine, and this pattern is not changed by protein deprivation. Furthermore, there is no change in the overall capacity to absorb valine. The authors stress, however, that the results are compared in relation to the surface area of the tissue used in the test, and it must be remembered that total mucosal area of protein deprived intestine is considerably less than that of control animals.

Second, the uptake of protein through the small intestine into the circulation was measured in both protein deprived and age-matched control rats. When the rats are given BSA

orally, greater quantities are found in the serum of protein deprived animals compared to controls (Worthington, Boatman and Kenny, 1974). This finding was confirmed and extended to show that malnourished rats transport approximately twice the amount of protein per gram body weight than controls over a thirty-fold range of doses of protein given (Rothman, Latham and Walker, 1982(a)). In addition, the increased amounts of protein in the circulation of protein deprived rats are not due to delayed clearance of antigen by the reticuloendothelial system (Rothman et al., 1982(b)).

It appears, therefore, that protein deprivation alters the barrier function of the mucosa, and leads to increased intestinal permeability. Although the mechanism(s) responsible for this situation are unknown, one suggestion is contained in the work of Worthington and Syrotuck (1976). They examined uptake of large molecular weight particles through the jejunum of protein deprived and normal rats by electron microscopy. Uptake of particles by both sets of rats is by pinocytosis, however, after 4 months deprivation, particles appear in spaces between the epithelial cells in protein deficient rats only. Deterioration of the junction between enterocytes (and possibly the basement membrane) may be one reason for allowing increased uptake of material from the gut into the circulation in protein deficient animals.

In summary, protein deprivation may result in several

changes in the morphology and function of the gastro-intestinal tract. These changes may act to preserve the integrity of the intestine by maintaining many of the functions at a reduced energy cost. However, with increasing severity and duration of deprivation, the deterioration of the mucosa becomes overwhelming, and the vital functions of cell turnover, and the digestion and absorption of nutrients, are impaired.

CHAPTER 2

THE IMMUNOLOGICAL CONSEQUENCES OF FEEDING ANTIGEN

INTRODUCTION

When antigen is first encountered via the oral route, a number of different immunological consequences may occur. These consequences differ according to factors such as the nature and dose of the antigen ingested, the age, species, and strain of animal involved, and the immune response examined.

Both systemic antibody and CMI responses have been observed after oral administration of antigen. For example, feeding BSA to NZW rabbits or to human infants can result in a systemic antibody response to the antigen (Rothberg, Kraft and Farr, 1967; Rothberg, 1969). Similarly, feeding schistosome egg antigen to mice can produce a systemic cellular hypersensitivity response (Perrotto, Hang, Isselbacher and Warren, 1974). Alternatively, local antibody responses, predominantly of the IgA isotype, are known to occur following oral immunization with antigen (data reviewed by Bienenstock and Befus, 1980).

More widespread attention, however, has been given to the suppression occurring in both limbs of systemic immunity which can be induced by feeding: the phenomenon of oral tolerance. This chapter will briefly review some of the mechanisms involved in the induction and maintenance of this tolerance, and the consequences for the small intestine of a disturbance in normal regulatory function.

ORAL TOLERANCE

The down-regulation of systemic immune responses which results from initial mucosal presentation of antigen has been recognised for many years. Over one hundred and fifty years ago, Dakin (1829) reported a practice among North American Indians of ingesting poison ivy leaves to prevent dermatitis upon subsequent contact with the plant. Wells and Osborne (1911) demonstrated that the anaphylactic response of guinea pigs towards hen egg white or corn protein could be prevented by feeding the antigen before parenteral immunization. However, over the past decade, the phenomenon of oral tolerance has received considerable attention, and has been confirmed for different types of antigens, including contact sensitizing agents (Asherson, Zembala, Perera, Mayhew and Thomas, 1977), ovalbumin (Miller and Hanson, 1979; Mowat, Strobel, Drummond and Ferguson, 1982), and for heterologous red blood cells (Mattingly and Waksman, 1978; Kagnoff, 1978(a)).

Both humoral and cell mediated limbs of the immune response can be suppressed after feeding antigen, and this state of tolerance can be influenced by several factors including the dose of antigen, frequency of administration and the timing of feeding relative to challenge with the antigen (Bienenstock and Befus, 1980).

Systemic tolerance may also be accompanied by a mucosal antibody response, and this has been demonstrated by Challacombe and Tomasi (1980), who have shown a local

salivary IgA response occurring simultaneously with suppression of both systemic antibody and CMI responses. This conclusion is supported by the finding that a single antigen feed results in the presence in the Peyer's patches of both T_h cells for mucosal IgA responses and T_s cells for systemic IgG responses (Richman, Graeff, Yarchoan and Strober, 1981). However, this situation has recently been called into question (Elson and Ealding, 1984). Using the potent oral immunogen, cholera toxin, together with the less potent antigen, keyhole limpet haemocyanin (KLH), these authors have shown that intestinal IgA responses and tolerance for systemic antibody responses are reciprocally linked, and do not occur simultaneously. The conflict in the conclusions between these reports may arise from differences in the type of antigen used, the site of mucosal antibody sampled (intestinal vs. salivary), and also from the fact that multiple feedings of antigen are used in the latter report.

Many different mechanisms have been implicated in the control of oral tolerance. Serum factors from orally immunized mice have been shown to suppress the immune response both *in vivo* and *in vitro*. Circulating antigen: antibody complexes (possibly IgA complexes) are capable of suppressing humoral immune responses (André, Heremans, Vaerman and Cambiaso, 1975), and the IgG fraction of serum from fed mice is also suppressive when added to *in vitro* antibody

response cultures (Chalon, Milne and Vaerman, 1979). Kagnoff (1978(b)) has suggested that part of the suppressive activity of the antigen fed serum is mediated by anti-idiotypic antibody. All of these reports are comparable, in that the serum suppressive factor is the direct result of a response to the fed antigen. The report of Strobel and his colleagues is unique, as they have identified a factor which is present in the bloodstream of mice only one hour after feeding, which, when transferred to naive syngeneic recipient mice, can specifically suppress the DTH response to the antigen in question (Strobel, Mowat, Drummond, Pickering and Ferguson, 1983). This factor is the product of intestinal processing of antigen, and can activate a population of T_s cells capable of suppressing DTH.

Cellular control mechanisms have been extensively demonstrated in oral tolerance. While B suppressor cells are present in lymphoid organs of mice fed contact sensitizing agents (Asherson et al., 1977), T cell mediated suppression seems to be the predominant mechanism for inducing hypo-responsiveness to a variety of antigens (Asherson et al., 1977: Mattingly and Waksman, 1978; Richman, Chiller, Brown, Hanson and Vaz, 1978; Miller and Hanson, 1979). Adoptive transfer of cells from antigen fed animals into naive syngeneic recipients demonstrates that the T_s cells inhibit both limbs of the systemic immune response, and that these cells act on the afferent phase of the CMI response, as they are effective only when transferred before, or shortly

after, the time of sensitization (Miller and Hanson, 1979). The immune status of B cells within orally tolerized hosts remains a point of debate, with different reports showing that B cells either retain (Titus and Chiller, 1981), or lose (Vives, Park and Weigle, 1980) their functional activity. Suppressor cells appear in the Peyer's patch and mesenteric lymph nodes of antigen fed mice sooner than they do in the spleen, suggesting that migration of these cells after sensitization in local sites may occur (Mattingly and Waksman, 1978).

In vitro studies have allowed workers to examine the complex series of events which occur during oral tolerance. Antigen feeding appears to activate a feedback suppressor circuit in the spleens of mice in which Lyl^+ 23^- $I\text{-}J^+$ T cells induce normal T cells to become suppressor effector cells (MacDonald, 1982). The suppressor inducer cell which initiates the suppressor circuit in the spleen migrates from the Peyer's patches shortly after antigen exposure (Mattingly, 1984). In addition, the existence of a contrasuppressor circuit within Peyer's patches which may allow for a local immune response to occur within the framework of systemic tolerance, has been proposed (Green, Gold, Martin, Gershon and Gershon, 1982).

Active suppression of the immune response by T_s cells is the most widely studied mechanism for the induction of tolerance. It is also important to note, however, that feeding antigen may also cause direct anergy of T_h cells.

This has been demonstrated directly by Titus and Chiller (1981), and also by Vives et al. (1980). Furthermore, T_h cell anergy is likely to be the reason behind the suppression in cyclophosphamide treated, antigen fed mice from the work of Hanson and Miller (1982). The different mechanisms for oral tolerance are not mutually exclusive. Indeed, at high doses of antigen ingested, it is likely that several mechanisms may be important to induce and maintain the appearance of suppression (Mowat et al. 1982).

Feeding antigen after immunization can substantially alter the pattern of the antibody response. In mice previously immunized by a parenteral injection of antigen in adjuvant, oral antigen exposure results in a secondary antibody response (Hanson, Vaz, Rawlings and Lynch, 1979). In contrast, Lafont and his colleagues have demonstrated that feeding after immunization can prevent a secondary response, and interrupt IgE production (Lafont, André, André, Gillon and Fargier, 1982). This effect is dependent upon repeated feedings of antigen, and the quality of results is dependent upon the strain of mouse used. Furthermore, the suppressive effect of post immunization feeding is different according to which isotype is examined ; the IgE response is easily suppressed after i.p. priming, the IgG response is unaffected, and the IgA response is enhanced by feeding after priming (Saklayanen, Pesce, Pollak, and Michael, 1984).

Finally, other factors can be important in determining the quality of tolerance observed after feeding. The

differences which exist in oral tolerance induction between mice of different inbred strains indicate that the genetic makeup of the host influences this phenomenon (Stokes, Swarbrick, and Soothill, 1983). The stage during ontogeny at which the antigen is first encountered orally is also an important factor. When the feeding occurs within the first week of life, priming and not tolerance for subsequent humoral and cell mediated responses is found (Hanson, 1981; Strobel and Ferguson, 1984).

In conclusion, a number of different mechanisms have been shown to be active in the induction and maintenance of oral tolerance, and it is likely that in each experimental system, different mechanisms are predominant. The next section will discuss methods which have been used to alter the induction of tolerance, and the consequences for the gut of a breakdown in normal regulatory mechanisms.

ABNORMAL REGULATION OF MUCOSAL IMMUNITY

Several methods have been used to impair the induction of oral tolerance. When the drug cyclophosphamide (CY) is administered 2 days before feeding, tolerance for systemic antibody and DTH responses is partially impaired (Mowat et al, 1982). This observation is related to the finding that CY selectively depletes a population of T_s cells (Askenase, Hayden, and Gershon, 1975). The fact that tolerance is not completely abrogated in these experiments can be interpreted to suggest that several suppressive mechanisms are activated by feeding.

Stimulation of the reticuloendothelial system (RES) using oestradiol and muramyl dipeptide (MDP), has been shown to impair oral tolerance to OVA (Mowat and Parrott, 1983; Strobel, 1983). Induction of a mild GvHR in F_1 animals can also prevent tolerance, and it has been shown that this is associated with an enhanced ability of splenic antigen presenting cells (APC) to present OVA to effector T cells (Strobel, Mowat, and Ferguson, 1985). Preliminary data indicate that oestradiol and MDP share this effect on APC (Mowat, personal communication). It is not known however, if the prevention of tolerance induction observed in these cases is accompanied by a decreased induction of T_s cells. Evidence supporting this hypothesis comes from a report by Yoshikai and coworkers, who have shown that stimulation of the mononuclear phagocyte system by diethylstilbrestol results in decreased T_s cell activity to a parenteral injection of SRBC (Yoshikai, Miake, Matsumato, Nomoto, and Takeya, 1981).

In conditions where oral tolerance for DTH responses is impaired, a CMI response can be induced in the intestine following challenge with antigen. Due to the inaccessibility of this organ, the response is assessed indirectly by measuring structural changes in the gut mucosa, and these changes take the form of increases in the crypt cell production rate (CCPR), the crypt depth, and the number of IELs within the villous epithelium (Mowat and Ferguson,

1981(a)). This situation is observed with CY, oestradiol, and MDP, where pretreatment of the host with these agents before feeding antigen allows for the development of a DTH response in the gut following continuous oral challenge with the antigen (Mowat and Ferguson, 1981(a); Mowat and Parrott, 1983; Strobel, 1983). In the case of CY, the response develops because administration of the drug leads to elimination of an important suppressor cell system which normally acts to prevent damaging DTH reactions occurring to dietary proteins at the level of the gut.

The fact that similar morphological changes are observed in the gut of patients with certain food hypersensitivity disorders e.g. cow's milk protein intolerance and coeliac disease, suggests that they may be caused by an active CMI response at the gut mucosa. It appears, therefore, that oral tolerance to dietary proteins may be an important physiological mechanism which exists to prevent the development of damaging food sensitive enteropathies (Mowat and Ferguson, 1981(a)).

CHAPTER 3

MATERIALS AND METHODS

Animals

For the majority of the experiments, male and female BDF_1 mice ($H\text{-}2^{b/d}$) were used. These mice were bred in the Animal Unit of the Western General Hospital, Edinburgh by mating a male DBA/2 line ($H\text{-}2^{d/d}$) with a female C57BL/6J line ($H\text{-}2^{b/b}$). Female BALB/c mice ($H\text{-}2^{d/d}$) were also used in some experiments

Mice were weaned onto the experimental diets at 3 weeks of age.

Diets

The diets used in this work were manufactured to my specifications by Special Diet Services Ltd. (Witham, Essex) They contained 4% or 24% protein, were isocaloric, and ovalbumin-free. The formulation of the two diets supplied, and the composition of the vitamin/mineral premixes in both cases are contained in Tables 3.1 and 3.2. The source of protein in the 4% diet was from cereals and soya bean meal, and the 24% diet utilized cereals, soya bean and fish meal for its protein source. The animals were allowed unlimited access to the diets and tap water throughout the duration of experiments. Mice maintained on the 4% protein diet were also offered pieces of rind-less cucumber as an additional source of water.

In certain experiments, mice which were maintained on the standard laboratory diet (18.4% protein: Spratts No.1 Spratts Patent Ltd., Cambridge) were used as recipients of

cells. They were used in groups where there were insufficient numbers of 24% protein maintained mice.

Additionally, in experiments contained in Chapter 10, groups of mice were fed OVA or HSA, which was incorporated into their drinking water at a concentration of 2 mg/100 ml. Allowing for a water intake of approximately 5 ml/day, this was equivalent to a daily dose of 100 µg/mouse, and was of no consequence nutritionally.

Anaesthesia

Procedures such as retro-orbital bleeding and footpad injections were performed under light ether anaesthesia.

Body weights

Mice were weighed using an Oertling TP40 single pan balance.

Organ weights

Organs were dissected free of surrounding tissue, placed in a preweighed, air-tight container to prevent drying, then weighed on an Oertling R20 balance.

Antigens

Ovalbumin (OVA), human serum albumin (HSA) and bovine serum albumin (BSA) were obtained as five times recrystallised fractions from the Sigma Chemical Company (Poole, Dorset, U.K.). These antigens were dissolved in physiological saline (0.9% w/v:

Phoenix Pharmaceuticals, Gloucester, U.K.) before use.

Determination of total serum protein and serum albumin levels

Measurements of total protein and albumin in the serum of 4% and 24% protein mice were performed using diagnostic kits obtained from the Sigma Chemical Company (Poole, Dorset, U.K.). Protein levels were determined through the ability of copper to react with the peptide bonds of serum proteins, and albumin levels were determined as a consequence of direct binding of the dye, bromocresol green, to that protein.

Oral administration of antigen

Mice were fed OVA, dissolved in saline, via the oesophagus. With mice maintained on the 24% protein diet, a rigid stainless steel dosing needle with a spherical, blunded end was used. However, with 4% protein mice, it was necessary to use a premature infant feeding catheter (Argyle, Sherwood Medical Industries, 3.5 Charriére) attached to a shortened 19 gauge stainless steel needle, to prevent mucosal abrasion during intubation. This dosing needle was the kind gift of Dr Stephan Strobel.

Parenteral immunization of animals

All groups were immunized intradermally (i.d.) into one rear footpad with 100 μg of antigen, emulsified in complete Freund's adjuvant (CFA: H37Ra - Difco Ltd.). Total injection volume was 50 μl. When animals were used

as cell donors for passive transfer experiments, they received 100 μg antigen/CFA into each rear footpad.

Bleeding of mice

Approximately 200 μl of blood were obtained routinely from the retro-orbital plexus using heparinised haematocrit tubes (Propper Ltd., Long Island, New York). When larger quantities of blood were required e.g. in serum transfer experiments, mice were exsanguinated from the axillary vein. Haematocrit tubes were sealed with Cristaseal (Hawksley, England), and allowed to clot in an upright position. They were spun in a haematocrit centrifuge (Hawksley, England) for two minutes, the serum was removed, and it was stored at -20°C until assayed.

Cyclophosphamide treatment

In certain experiments, mice were given 100 mg/kg cyclophosphamide (CY: Endoxana Ltd.) in saline intraperitoneally (i.p.).

Assessment of systemic delayed-type hypersensitivity

Mice were tested for delayed-type hypersensitivity (DTH) responses by measuring the specific increment in footpad thickness 24 hrs after an i.d. challenge of 100 μg of antigen in 50 μl saline into the non-immunized rear footpad. The response is expressed as the difference in thickness (in mm) between the pre- and post-challenge footpad as measured by microcalipers (Pocotest A, Carobronze Ltd., London). Control mice were immunized with saline/CFA and

challenged with antigen.

ELISA method to detect IgM and IgG anti-OVA antibodies in mouse serum

The detection of IgM and IgG antibodies to OVA was performed by an ELISA method. OVA was dissolved in carbonate buffer (0.05 mol/litre pH 9.6: North East Biomedical Labs. Ltd.) to a final concentration of 10 μg/ml. 125 μl of this solution was added to each well of an EIA microtitre plate (Dynatech Labs. Ltd.), and the wells allowed to coat with the antigen for 16 hrs at 4°C in a moist box.

The plates were washed three times with washing buffer, and 125 μl of appropriate dilutions of the reference and test sera (1/50 dilution - IgM isotype ELISA; 1/400 dilution - IgG isotype ELISA) were added to the wells. The plates were then incubated for 2.5 hours in a moist box at room temperature, followed by a further washing stage. The next stage of the ELISA involved adding 125 μl of the goat anti-mouse Ig conjugated to alkaline phosphatase to each well, and incubating at room temperature for 3.5 hours. The conjugate antibody used in the IgG ELISA was directed against both heavy and light chain determinants and was diluted 1/5000 in serum diluent. For the IgM ELISA, the antibody was directed against heavy chain determinants and was diluted 1/2000 in serum diluent. Both conjugates were purchased as affinity-purified, isotype-specific antisera from Jackson Immunoresearch Labs., U.S.A.

125 µl of the substrate solution (1 mg/ml p-nitrophenyl-phosphate (Sigma) in 10% diethanolamine buffer (BDH Ltd.)) was added to each well and the plates incubated in a moist box at room temperature. The plates were read in an MR580 Microelisa autoreader (Dynatech Ltd.) at a wavelength of 405 nm, when the positive control reference sera had reached an absorbance value of 1.0. In the IgG ELISA, this happened after 15-20 mins. incubation but took up to 1 hour for the IgM ELISA.

The positive control reference sera for the IgG ELISA was pooled from mice immunized 21 days previously with 100 µg OVA/CFA, and the reference sera for the IgM ELISA was pooled from a different group of mice immunized 10 days previously. In each assay, conjugate controls (wells coated with antigen, containing conjugate substrate, but no serum) were included to determine the non-specific binding of the conjugated antiserum to the antigen coated wells, and the ELISA reader was zeroed against these wells. In addition, each plate contained wells using sera from non-immunized mice as negative reference sera. Samples were assayed in duplicate, and results expressed as individual absorbance readings.

The isotype specific ELISAs were developed in conjunction with Mrs M. Gordon.

The ELISA method used in Section A, Chapter 8 was developed prior to my arrival in the laboratory, and was performed under different conditions. The differences between the methods were as follows:-

Antigen coating concentration	-	1.0 mg/ml
Dilution of reference and test sera	-	1/100
Conjugate antibody	-	rabbit anti-mouse IgG conjugated to alkaline phosphatase (Miles-Yeda Ltd.) 1/1750 dilution
Incubation time after addition of conjugate	-	16 hours
Time to reading after addition of substrate	-	approximately 30 mins.

The results of the experiment using this old method of ELISA (Chapter 8, Section A) suggested the need to develop the test to measure both IgM and IgG isotypes. Additionally, the sensitivity was improved in the new methods by using affinity-purified conjugate antibodies to detect IgM and IgG.

ELISA for the detection of OVA in mouse serum

This method employed a double antibody sandwich ELISA technique to measure levels of OVA present in mouse serum after feeding. Specifically purified rabbit IgG anti-OVA antibodies (a kind gift of Dr D. Hanson) were diluted in carbonate buffer (pH 9.6) to a final concentration of 10 µg/ml. 150 µl of this solution was added to each well of an acrylic microtitre plate (Falcon, Becton Dickinson) and the wells allowed to coat with antibody for 2 hrs at 37°C. All incubation stages were performed with the plates in a moist box to prevent "edge effects". The plates were washed three times with washing buffer, and 125 µl of the OVA standards

(range $10^{0.5}$ - 10^{5} ng OVA in normal mouse serum) or the test sera (diluted 1/10 in serum diluent) were added to each well. The plates were then incubated for 16 hrs (overnight) at room temperature.

Following a further washing stage, 125 µl of rabbit IgG anti-OVA conjugated to alkaline phosphatase (1/500 dilution: North East Biomedical Labs. Ltd.) was added to the plates, and again left overnight at room temperature. The plates were washed three times and 125 µl of the substrate solution (1 mg/ml p-nitrophenylphosphate in 10% diethanolamine buffer) was added to each well. The plates were incubated at room temperature and were read at a wavelength of 405 nm once the 10^{5} dilution of the OVA standards had reached an absorbance value of 1.4. This event happened approximately 30 mins. after addition of the substrate solution.

Negative control wells which contained normal mouse serum with no ovalbumin, plus conjugate and substrate, were included in each plate, and the microelisa reader was zeroed against these wells. Each well was assayed in duplicate, and the readings from the OVA standard wells enabled a standard curve of absorbance values against ng OVA/ml mouse serum to be drawn. The amount of OVA present in the test sera could be read off from the standard curve. The lower limit of sensitivity of the assay in my hands was 10 ng OVA/ml serum, and absorbance readings giving values below this figure were discounted.

Sacrifice of animals

Mice were killed by cervical dislocation.

Removal of tissues

Organs were obtained for histology and microdissection immediately after sacrifice. Pieces of small intestine (approximately 5 mm x 5 mm) were removed, free of lymphoid aggregates. They were opened, placed on card, and immersed in fixative. Samples of small intestine containing Peyer's patches were also opened and placed on card before fixation. Spleen and liver samples were trimmed with a razor blade before being placed into fixative.

Fixatives and histology

For conventional histology, small intestine specimens, Peyer's patches, spleen and liver were fixed in 10% buffered formalin. Thymus, mesenteric and peripheral lymph node specimens were fixed in Bouin's fluid. They were embedded in paraffin wax, and sections 5 μm thick were cut. The tissues were stained with haematoxylin and eosin, before examination using either a Leitz ortholux II or a Leitz dialux EB20 microscope.

Histological processing was carried out by Mrs Linda McCardle and Mr Alexander Sutherland.

Intraepithelial lymphocyte counts

Intraepithelial lymphocytes (IEL) were counted by the method of Ferguson and Murray (1971), and are expressed as the number of IEL/100 epithelial cells. Specimens were examined under x 1000 (oil immersion) magnification, and only sections with a single epithelial cell layer were counted. Differential cell counts were performed by counting epithelial and lymphoid cell nuclei lying above the basement membrane, and a total of 600 cells were counted in each section.

Processing of tissues for microdissection

The method of Clarke (1970) was used. Mice were injected with 7.5 mg/kg colchicine (BDH Ltd.) i.p., and were sacrificed at intervals of 15-90 mins thereafter. Pieces of small intestine were removed, cut open, and placed on card prior to being fixed in Clarke's fixative for a maximum of 24 hrs. After this, tissue was stored in 75% ethanol before being used for microdissection. To allow for repeated examination, only one half of the tissue was stained by the modified Feulgen reaction. The pieces of gut were immersed in 50% ethanol for 10 mins., followed by 10 mins in tap water, before being placed in 0.1N HCl at 60°C for 7 mins. After a further 10 mins in tap water, the specimens were rinsed with three changes of tap water, and then stained with Schiff reagent (Difco Ltd.) for 30 mins. at room temperature. The specimens could then be stored in tap water for a maximum of 48 hrs before microdissection.

The tissue was examined under the dissecting microscope (x 32 magnification: Zeiss Stereomicroscope 4B) and the muscularis mucosa removed using fine forceps. Thin strips of mucosa each containing a few villi and surrounding crypts, were then cut from the edge by dissection with a cataract knife (Weiss Ltd.). These fragments were placed on a microscope slide in 45% acetic acid, covered with a coverslip, and examined under the microscope (Wild M20, Heerbrugg, Switzerland) with a previously calibrated eyepiece micrometer. From each specimen, the lengths of 10 representative villi and crypts were taken, and the means calculated for group comparisons. The pieces were then gently squashed under the coverslip and the number of metaphases per crypt counted for between 15-20 intact crypts.

To obtain the crypt cell production rate per hour (CCPR), the number of metaphases per crypt was paired with the corresponding time interval after colchicine injection, and the set of results were analysed using a linear regression programme on a Casio fx 180P calculator. Having established linearity, the CCPR was calculated from the gradient of the line of best fit, taken by the method of least squares.

Preparation of cell suspensions

Spleen, inguinal and popliteal lymph node specimens were removed from the animal immediately after sacrifice. They were dissected free of surrounding tissue in medium, and cut into smaller pieces. For spleen cell suspensions,

RPMI 1640 (Flow Labs., Irvine) was used, while for lymph node cell suspensions, Hank's balanced salt solution (HBSS: Gibco) was used. These specimens were gently passed through a fine gauge wire mesh using the plunger of a 5 ml syringe (B.D. Ltd.) and the resultant suspension transferred to a sterile universal bottle (Sterilin Ltd.). This was allowed to stand for a few minutes at room temperature to allow debris to settle out. The cell suspensions were then washed three times at 400 g with fresh medium. After the final wash, the cells were counted in a haemocytometer (improved Neubauer chamber) using white cell diluting fluid, and cell viability was assessed using the method of trypan blue exclusion. The final cell pellet was made up to the required concentration of viable cells using the appropriate medium.

Induction of a Graft-versus-Host Reaction

In all cases, female C57BL/6J mice were used as sources of donor spleen cells. Recipients were unirradiated BDF_1 mice. In experiments to determine the ability of spleen cells from protein deprived parental mice to induce a GvHR, F_1 mice received 4.5×10^7 cells in 0.2 ml medium i.p. Control mice received either an equivalent number of cells from normally fed parental mice or medium alone. In experiments which examined a GvHR induced in protein deprived F_1 mice, recipients received 6×10^7 cells in 0.2 ml medium i.p. Control mice received medium alone.

Assessment of Graft-versus-Host Reaction

The spleen weight assay of Simonsen was used (Simonsen, 1962). Mice were weighed prior to sacrifice, the spleen removed and weighed, and the relative spleen weight expressed as mg spleen/10 g body weight. The spleen index is given by the formula:-

$$\frac{\text{Mean relative spleen wt. in GvHR mice}}{\text{Mean relative spleen wt. in control mice}}$$

Mitogen stimulation of mouse lymphocytes

The method was developed for use in the laboratory by Dr M. Bruce, and employed the mitogen phytohaemagglutinin (PHA) to study the transformation of T lymphocytes in vitro.

Culture medium: RPMI 1640 medium (Flow Labs. Ltd.) supplemented with 2 m.mol/l glutamine (Flow Labs. Ltd.), 100 I.U./ml penicillin and 100 µg/ml streptomycin (Flow Labs. Ltd.), 50 µ.mol/l 2-mercaptoethanol (Sigma), 5% human AB+ serum, and buffered with 10 m. mol/l HEPES buffer at pH 7.2 (Flow Labs. Ltd.).

Mitogen: PHA (Wellcome) at a concentration of 0.45 mg/ml in the cultures.

Mice were killed and popliteal and inguinal lymph nodes were quickly removed. Cell suspensions were made as before in culture medium, and 5 x 10^5 viable cells in a volume of 200 µl was dispensed to each well of a flat-bottomed Microtiter plate (Flow Labs. Ltd.). Each sample was performed in

quadruplicate. PHA was added to the wells and the plates were incubated in a Hotpack humidified CO_2 incubator at 37°C with 80% humidity in a mixture of 5% CO_2 and 95% air. Empty wells around the side of the plate were filled with saline, and control wells were included in each plate which contained cells plus mitogen without radioactive label, cells only with label or cells only without label. The day of setting up the culture was designated day 0.

On day 2, 18 hrs. before the culture was due to be harvested, the appropriate wells were pulsed with radioactive label. Each well received 0.01 μCi of C^{14}-Thymidine diluted in sterile saline to a final volume of 5 μl. Unlabelled wells acted as controls for background radiation counts.

On day 3, the cells were harvested onto Titertek filter paper discs (Flow Labs. Ltd.) using an automated cell harvester (Dynatech Automash). The discs were allowed to dry and then placed in plastic minivials (Scintillation minivials, A. and J. Beveridge Ltd.) inside glass scintillation vials (A and J. Beveridge Ltd.). These were filled with 2.5 ml Koch-lite scintillation liquid (A. and J. Beveridge Ltd.) inside a fume hood and sealed. C^{14}-Thymidine uptake was measured as counts per minute (cpm) using a Packard Tri-Carb. Scintillation spectrotometer which measures β emission.

The mean background c.p.m. was calculated and subtracted from all counts before the mean c.p.m. of quadruplicate wells was calculated. A stimulation index (S.I.) was calculated

where appropriate from the formula:-

$$S.I. = \frac{\text{mean c.p.m. of stimulated wells}}{\text{mean c.p.m. of unstimulated wells}}$$

Assay of epithelial cell disaccharidase enzymes

Pieces of small intestine (approximately 5 mm x 5 mm) were removed after sacrifice, and weighed, before being stored at -20°C. The brush border enzymes, maltase, sucrase, lactase and trehalase were assayed by the method of Dahlqvist (1964), and the results expressed as m.mol of substrate hydrolysed/minute/g. wet weight of tissue at 37°C. Assays for disaccharidase activity were performed by Mrs M. Gordon and Mrs B. Walton.

Processing of tissues for scanning electron microscopy

Pieces of small intestine, containing a Peyer's patch were pinned out, villous surface upwards on top of a wax base. These specimens were fixed in 2.5% glutaraldehyde in cacodylate buffer pH 7.3 for 24 hr. at 4°C. After a further wash in cacodylate buffer, the specimens were dehydrated through a graded series of alcohols up to 100%, and were then subjected to critical point drying in CO_2 using acetone as the transmission medium. They were mounted on aluminium stubs, sputter-coated with gold to a depth of 20 nm, and were examined on an ISI 60 Scanning Electron Microscope. Processing of the tissues was carried out by Miss J. Tocher.

Statistical evaluations

In general, results are presented as the mean $\pm$ 1 standard deviation (s.d.) as stated in the figures and tables. A student's t-test was used to compare differences between group means.

Antibody levels, as measured by ELISA are expressed as optical density readings at 405 nm and are plotted individually on a graph. As a non-parametric distribution of this data was likely, a Wilcoxon rank sum test was used to compare differences in antibody levels between groups.

In both types of tests, a P value of > 0.05 was taken as not significant, a value of < 0.05 was taken as significant, and a value of < 0.01 was taken as highly significant. For reasons of simplicity, significance values of < 0.001 were recorded as being < 0.01 (highly significant).

Where appropriate (e.g. DTH responses in tolerance experiments), results are expressed as % suppression of control values given by the following formula:-

$$\% \text{ suppression} = \left(\frac{\text{control value} - \text{experimental value}}{\text{control value}}\right) \times 100$$

CCPRs, calculated by linear regression, were compared by covariance analysis, to detect differences between the slopes. In practice, this test proved to be very stringent, and significant differences between CCPR values of control and experimental groups were found on very few occasions. All calculations were performed on a Casio fx 180 P calculator.

Solutions and buffers

ELISA reagents

Carbonate buffer:	0.05 mol/litre at pH 9.6 (North East Biomedicals Ltd.).
Washing solution:	0.05% Tween 20 (BDH Ltd.) in physiological saline.
Serum diluent:	0.05% Tween 20 in physiological saline with 0.2% sodium azide (BDH Ltd.).

10% diethanolamine buffer:

100 ml DEA (diethanolamine: BDH Ltd.).
800 ml distilled water
0.1015 g $MgCl_2.6H_2O$ (BDH Ltd.).
0.2 g sodium azide
pH 8.6

10% buffered formalin (1 litre):

Formaldehyde	100 ml
Distilled water	900 ml
$NAH_2PO_4.2H_2O$	4 g
Na_2HPO_4	6.5 g

Bouins fluid (1.05 litres):

Saturated aqueous picric acid (2,4,6-trinitrophenol) -	750 ml.
Formaldehyde	250 ml.
Glacial acetic acid	50 ml.

Clarke's fixative (1 litre):

Ethanol	750 ml.
Glacial acetic acid	250 ml.

	4% protein	24% protein
Barley	-	15.0%
Maize	20.0%	20.0%
Wheatfeed	-	25.0%
Full fat soya bean	3.0%	25.0%
Fish meal	-	10.0%
U.D. yeast	-	2.5%
Corn oil	5.0%	-
Cornflour	52.0%	-
Cereal fibre meal	15.0%	-
Vitamin/mineral premix	5.0%	2.5%

Table 3.1: Formulation of the protein-deprived (4%) and normal (24%) diets used.

	4% protein	24% protein
Vitamin A	5000 I.U.	5000 I.U.
Vitamin D_3	1000 I.U.	1000 I.U.
Vitamin E	50.0 mg	50.0 mg
Vitamin B_2	10.0 mg	8.0 mg
Vitamin K	10.0 mg	10.0 mg
Nicotinic Acid	20.0 mg	20.0 mg
Pantothenic Acid	20.0 mg	20.0 mg
Folic Acid	0.5 mg	-
Vitamin B_1	20.0 mg	20.0 mg
Vitamin B_6	20.0 mg	15.0 mg
Vitamin B_{12}	20.0 mg	18.0 mg
Lysine	400.0 mg	100.0 mg
Methionine	400.0 mg	100.0 mg
Choline Chloride	400.0 mg	400.0 mg
Iron	20.0 mg	20.0 mg
Cobalt	0.5 mg	0.5 mg
Manganese	70.0 mg	60.0 mg
Copper	10.0 mg	10.0 mg
Zinc	20.0 mg	10.0 mg
Iodine	0.5 mg	0.5 mg
Magnesium	1000.0 mg	-
Sodium Chloride	4000.0 mg	2000.0 mg
Phosphorus	7000.0 mg	1000.0 mg
Calcium	8800.0 mg	3000.0 mg

Table 3.2: Composition of the vitamin/mineral premixes used in the protein-deprived (4%) and normal (24%) diets. Figures represent amount per kilogram of diet.

CHAPTER 4

ESTABLISHMENT OF A MOUSE MODEL OF PROTEIN DEPRIVATION

Introduction to experiments

In order to examine the effect of protein deprivation on the induction and expression of intestinal immunity, it was first necessary to establish a reproducible animal model with which to perform the relevant experiments. The results detailed in this chapter were obtained during the establishment of this model.

The weights and physical appearance of protein deprived mice were assessed over a period of weeks, and compared to those of age-matched control mice maintained on the protein sufficient diet. Other measures used to indicate the extent of malnutrition were the total protein and albumin levels in the serum, and the packed cell volume (PCV) in blood, which acts as an indicator of haemoglobin concentration. Mice of a different genetic background were subjected to protein deprivation to examine if differences existed between the mouse strains in their ability to cope with a reduced protein intake. Finally, the problems which were encountered with this model, and the strategies used to attempt to solve them, are described.

General experimental design

Male and female BDF_1 mice aged 3 weeks were randomly assigned into groups, and were weaned onto the isocaloric diets described previously. The mice were allowed unlimited access to the diets and tap water for the duration of the experiment. The animals were examined regularly, and were

weighed once per week. In one experiment, female BALB/c mice, aged 3 weeks, were used.

Physical appearance of normal and protein deprived BDF_1 mice

Mice maintained on the 24% protein diet showed a steady rate of growth from weaning until they were about 6 or 7 weeks old. After this time, it was impossible to discern any change in size at all. The coat of the normal mice was jet black in colour, and was smooth and shiny (Figs. 4.1, 4.2). The mice were very active - a property associated with a few F_1 hybrid strains - and often proved difficult to handle.

In contrast, mice maintained on the 4% protein diet did not appear to grow in size until the third week after weaning, when a slow rate of growth was observed. However, at all times during the experiments, protein deprived mice appeared smaller than age-matched, normal diet, control mice. The coat of these mice was matt black, with no bald patches. The hair appeared to be slightly erect, giving the coat a rough texture. This difference was most noticeable after short periods of protein restriction (e.g. 2 weeks), in contrast to longer periods (e.g. 6 weeks), where this difference was barely apparent (Figs. 4.1, 4.2).

Protein deprived mice adopted a crouched posture with a curved spine, but remained as active as normal mice. Furthermore, when attempting to handle them, the mice became very agitated, more so than 24% protein mice, and often leapt

from the cage when the lid was removed. At no point during the experiment did the protein deprived mice develop diarrhoea.

Weights of normal and protein deprived BDF_1 mice

Figure 4.3 shows the weights of male and female BDF_1 mice which were maintained on the diets for a total of 12 weeks from weaning. Each point represents the mean of 4-8 mice.

It can be seen that male and female mice on the 24% protein diet gained weight rapidly from weaning, with male mice gradually attaining a higher weight. With both male and female mice on the 4% protein diet, there was no loss or gain of weight until 5-6 weeks after weaning, where both sexes gained weight slowly until the end of the experiment. There was no difference in weight between protein deprived male or female mice at any time.

The effect of short term protein deprivation initiated at weaning or maturity on BDF_1 mouse weights

This trial was intended to examine the ability of protein deprived female mice to gain weight after being placed on the normal protein sufficient diet. In this case, protein deprivation was initiated both at weaning and also once the mice had reached maturity. When 2 weeks restriction was begun at weaning, the mice failed to gain weight initially (Fig. 4.4). However, when offered the normal diet, they

rapidly gained weight to a level equivalent to age-matched mice on the 24% protein diet throughout (e.g. compare with Fig. 4.3). This effect of rapid weight gain after nutritional rehabilitation was noticeable even after longer periods (e.g. 10 weeks) of protein deprivation (data not shown). When 2 weeks restriction was begun at maturity, the animals lost weight at a constant rate over the restricted period (Fig. 4.4). However, once switched to the 24% protein diet, they immediately started to gain weight, and reached levels equivalent to those of age-matched 24% protein maintained mice after a further 4 weeks on the normal diet (e.g. compare with Fig. 4.3).

Presence or absence of anaemia in protein deprived BDF_1 mice

Female BDF_1 mice were protein deprived for different periods of time, and the changes in packed cell volume (PCV) in the blood were monitored. The results in Table 4.1 indicate that 2 weeks protein deprivation decreased the PCV in blood compared to controls ($P < 0.01$). After 6 weeks, however, there was no difference ($P > 0.05$) between the levels for 4% or 24% protein mice, but after 12 weeks, protein deprived mice again had decreased PCV in their blood ($P < 0.05$).

Serum protein analysis of protein deprived BDF_1 mice

Serum samples were removed from the same group of animals that were used in the last section, and the values for total protein and albumin concentration were estimated. Total protein levels were consistently decreased in protein deprived

mice at the time points examined (Table 4.1: $p < 0.01$ compared to control mice). Albumin levels were decreased only after 6 weeks protein deprivation ($p < 0.05$), but were normal after both 2 and 12 weeks deprivation compared to control mice ($P > 0.05$).

Physical appearance and weight of protein deprived BALB/c mice

The ability of mice from a different inbred strain to cope with a reduced protein intake was examined by maintaining female BALB/c mice on the 4% protein diet for 6 weeks from weaning. Control mice were fed the 24% protein diet.

In contrast to the situation observed with BDF_1 mice, protein deprived BALB/c mice appeared to grow directly from weaning, although always remaining smaller than the 24% protein control mice. They showed no difference in the condition of their coat and they adopted a normal posture. In addition, the mice were not hyperactive when attempting to handle them. Confirmation of the growth of protein deprived mice from weaning was obtained from the graph of body weights vs. time after weaning (Fig. 4.5). There was no difference between the weights of normal and protein deprived mice at weaning ($P > 0.05$), however, thereafter protein deprived mice had a reduced body weight compared to control mice at all times examined ($P < 0.05$).

Problems associated with the model of protein deprivation

The manufacturers of the diets had stated at the beginning that because of the reduced protein content of the 4% protein diet, they might find it difficult to pellet the diet into cubes. This, in fact, proved to be the case, with the pellets that were provided being very fragile and easily crumbled into powder. This therefore meant that during transport, storage and when the diet was provided in the feeding tray of the cage, much of it was lost as powder in the feeding bin and mixed with the wood shavings in the bottom of the cage. In an effort to cut down on this wastage, when the supplies of the diet were low, the powder was mixed with water to form a paste, and this was offered to the protein deprived mice in a small bowl. This made no difference to the palatibility of the 4% protein diet, and the mice were equally happy with the diet in both paste and pellet form.

The most major problem occurred during the first set of experiments, where the mortality rate of protein deprived mice was as much as 40%. The reason for this situation was unknown, however, it was observed that the dead mice were very much smaller than littermates in the same cage who existed quite happily on the 4% protein diet. It was reasoned that each animal, in order to survive the first few weeks of life, had to consume a considerable amount of food. As a consequence of large numbers of animals in small cages, and the resulting competition for food, many animals would not receive their fair share of 4% protein diet. These animals would then fail to thrive, and would eventually weaken and die. This high

mortality rate was not observed in control cages, because even those animals which ate less diet than others would receive sufficient quantities of protein to survive and grow. For this reason, protein deprived mice were housed under the following conditions to reduce the competition for diet:-

maximum 3 mice - cage dimensions 28 cm x 12 cm x 12 cm

maximum 4 mice - cage dimensions 42 cm x 12 cm x 12 cm

maximum 8 mice - cage dimensions 42 cm x 22 cm x 12 cm.

This strategy reduced the mortality rate to $< 10\%$ for the remaining experiments. Under these conditions, one group of four mice have existed on the 4% protein diet for more than one year with no losses.

Where deaths occurred in cages, and the bodies were not immediately removed, the remaining mice often ate all the carcass, including the hair and bones. As this represented a considerable amount of protein ingested, the occupants of this cage were excluded from all experiments.

Concluding remarks

The state of protein deprivation induced in BDF_1 mice using the 4% protein diet has been monitored through changes in weight and physical appearance, and additionally through changes in blood packed cell volume and serum protein levels.

Protein deprived animals failed to gain weight initially, but after longer periods of deprivation, their weight did increase. Nutritional rehabilitation with a normal protein sufficient diet rapidly reversed the adverse effect of protein deprivation, and initiated normal growth patterns. This happened regardless of whether restriction was begun at

weaning or at maturity. Interestingly, the effect of protein deprivation was not as severe when BALB/c mice were used in the experiment. Finally, total serum protein levels were consistently decreased in deprived mice. This model will be used in the experiments described in this thesis.

Weeks from weaning	Diet	Packed cell volume (%)	P	Total serum protein (g/dL)	P	Serum albumin (g/dL)	P
2	4% protein	44.0 ± 1.3	<0.01	4.43 ±0.38	<0.01	3.14 ±0.30	n.s.
	24% protein	47.3 ± 1.8		5.49 ±0.25		3.38 ±0.32	
6	4% protein	42.5 ± 5.4	n.s.	4.43 ±0.48	<0.01	2.72 ±0.37	<0.05
	24% protein	46.9 ± 1.7		5.32 ±0.18		3.19 ±0.24	
12	4% protein	45.5 ± 2.8	<0.05	5.11 ±0.25	<0.01	3.62 ±0.43	n.s.
	24% protein	49.2 ± 0.4		5.71 ±0.31		3.73 ±0.58	

Table 4.1: Estimations of packed cell volume, total serum protein, and serum albumin levels in protein deprived and normal BDF_1 mice. The figures represent the mean ± 1 s.d. of 6-8 mice.

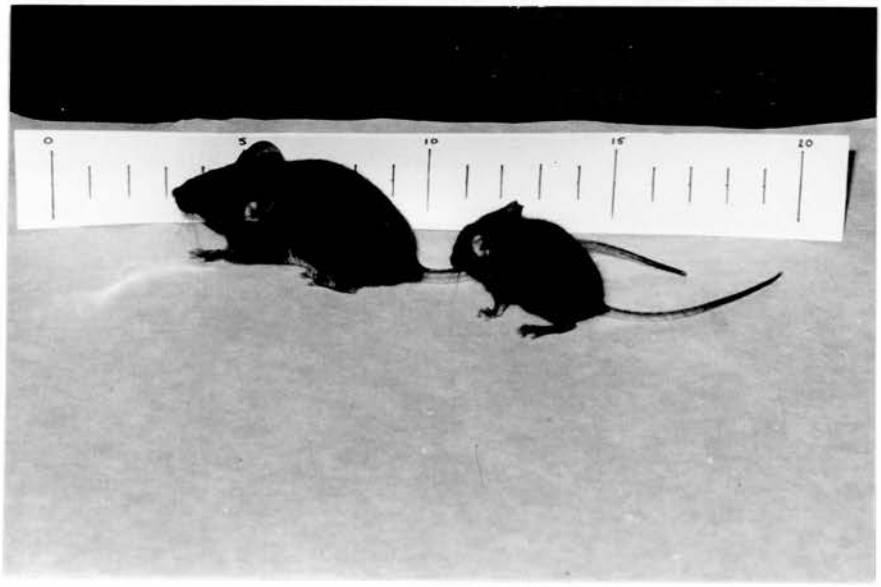

Fig. 4.1: A comparison of age-matched BDF_1 female mice maintained on the 4% or 24% protein diet for 2 weeks from weaning. Scale is in cm. The difference in size between the two mice can clearly be seen.

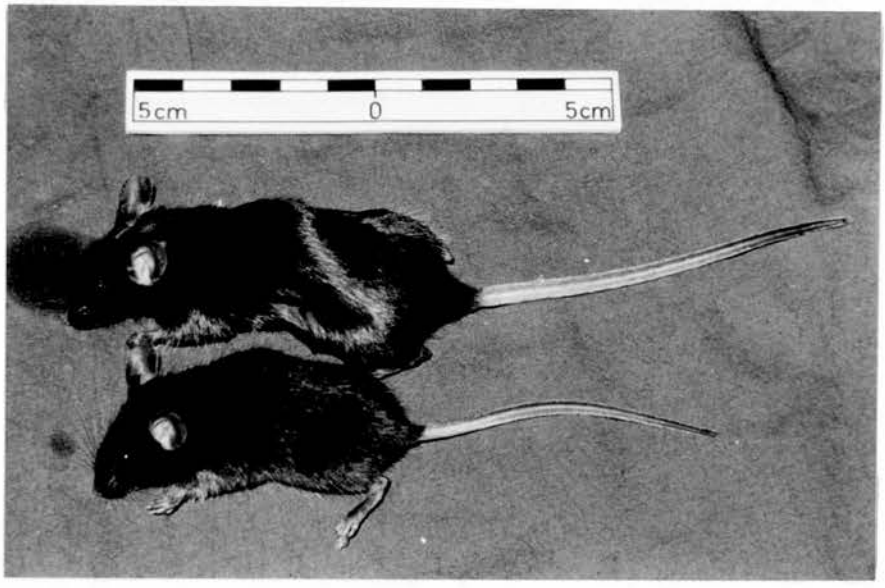

Fig. 4.2: A comparison of age-matched BDF_1 female mice maintained on the 4% or 24% protein diet for 6 weeks from weaning. The scale is in cm.

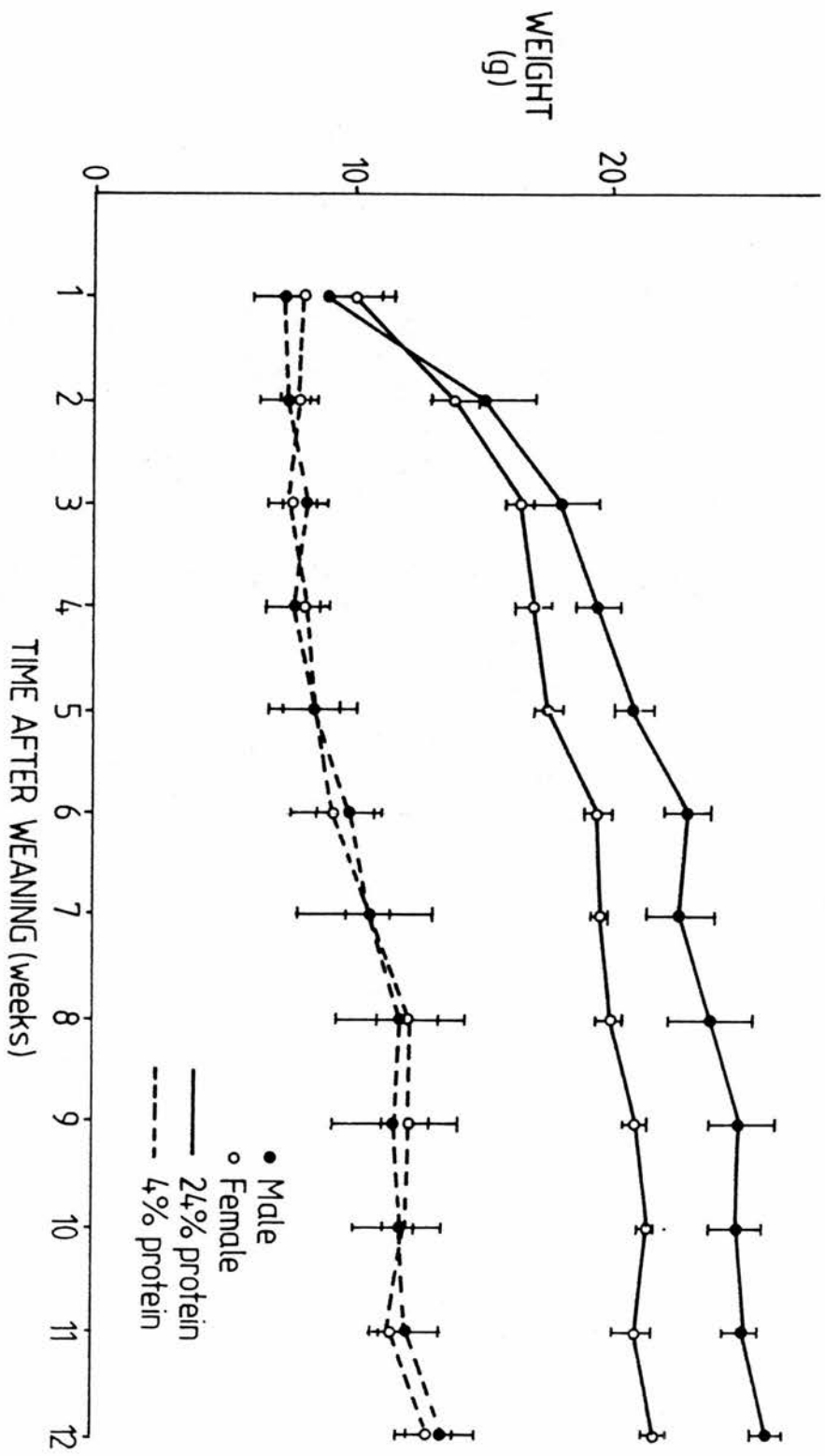

Fig. 4.3: Weights of male and female BDF_1 mice maintained on the 4% or 24% protein diet for 12 weeks from weaning. Each point represents the mean $\pm$ s.d. of 4-8 mice.

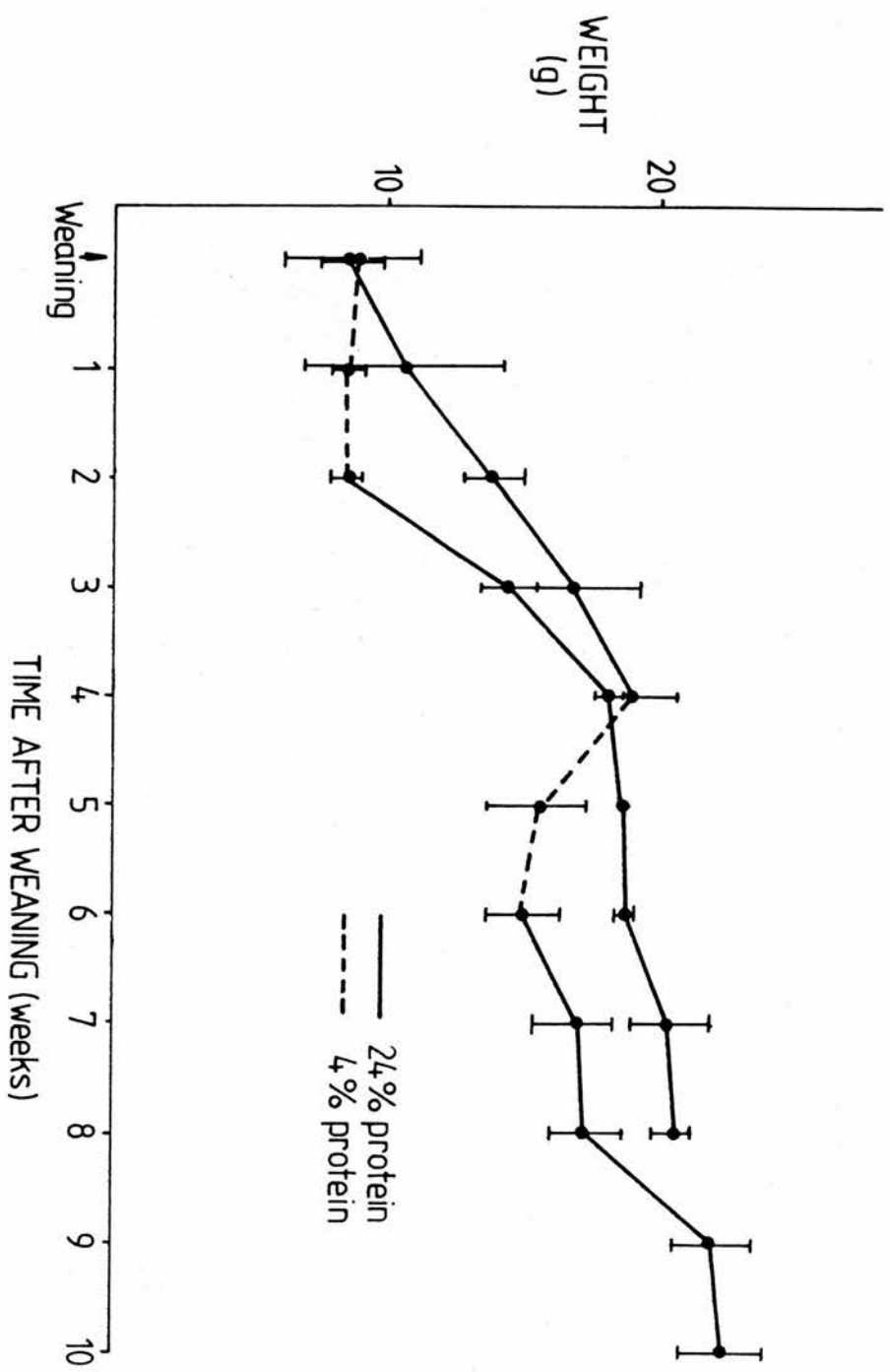

Fig. 4.4: Effect of 2 weeks protein deprivation initiated either at weaning or at maturity on the weights of female BDF_1 mice. Each point represents the mean $\pm$ s.d. of 7-9 mice.

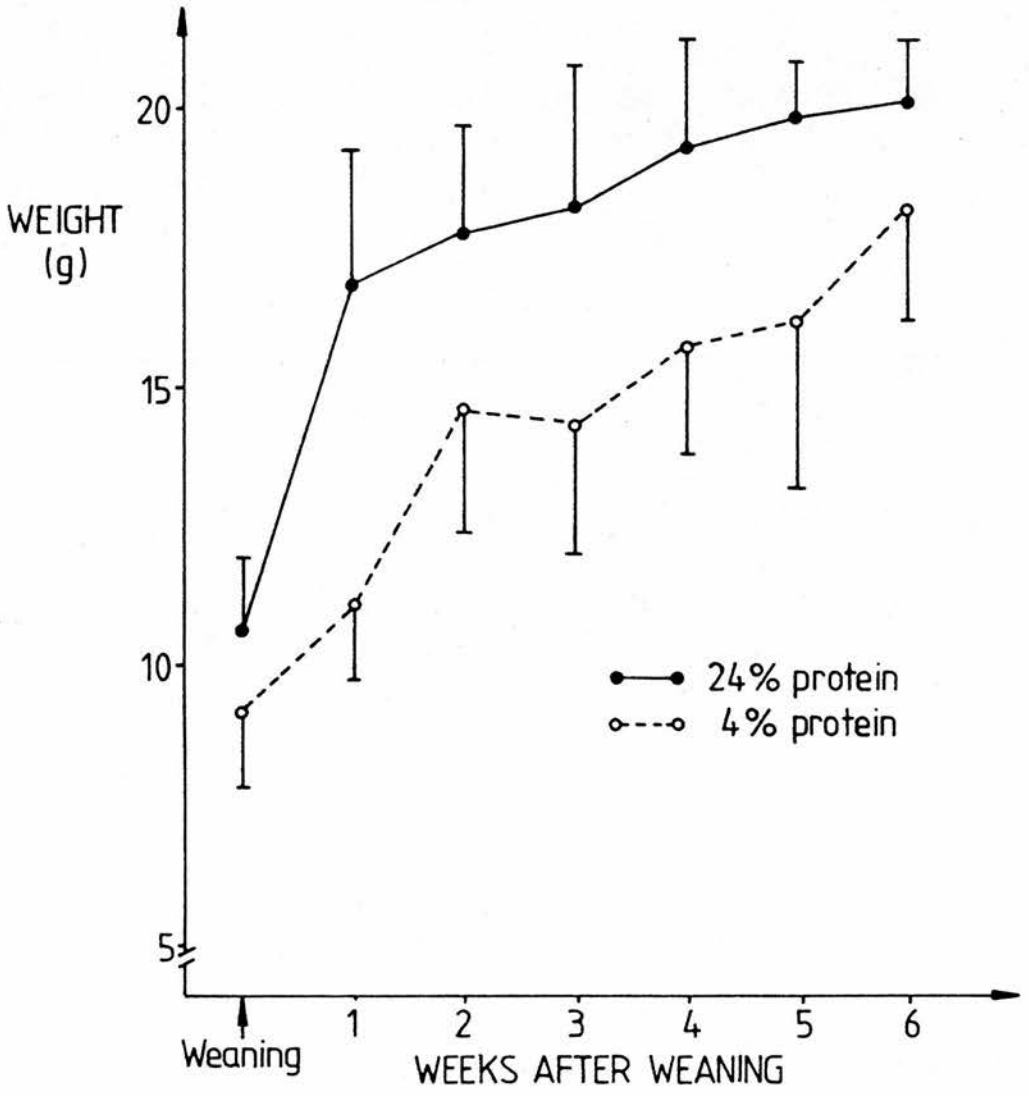

Fig. 4.5: Weights of female BALB/c mice maintained on the 4% or 24% protein diet for 6 weeks from weaning. Each point represents the mean $\pm$ 1 s.d. of 6-8 mice.

CHAPTER 5

STRUCTURE AND FUNCTION OF THE SMALL INTESTINE IN PROTEIN DEPRIVED MICE

Introduction

Many studies have investigated the effects of protein deprivation on small intestinal structure and function (see Chapter 1). One such study has reported that the structural alterations induced in the mucosa by protein deprivation are not consistent throughout the duration of the period of deprivation; for example, although the villus height of deprived rats is reduced after 4 weeks compared to control rats, it is normal when measured 1 or 10 weeks after initiation of the low protein diet (Syme, 1982). The aim of this section was to examine structure and function of the small intestine in the model of protein deprivation in use. In future experiments (Chapter 10), changes in gut structure are used to define the presence of a CMI response. Therefore, before protein deprived mice could be used in these studies, it was first necessary to define the baseline effect of different periods of deprivation on mucosal structure.

Experimental design

Groups of female BDF_1 mice aged 3 weeks were weaned onto diets containing 4% or 24% protein, and were maintained on these diets for a period of 2, 6 or 12 weeks. After this time, the animals were sacrificed, and samples of gut were removed for microdissection analysis and for determination of disaccharidase enzyme levels. The enzymes assayed were sucrase, lactase, maltase and trehalase, and this test is used routinely in the assessment of mucosal function. The

positions along the intestine at which samples were taken were chosen arbitrarily, and were within the first 2 cm from the pylorus for duodenum, within 10-15 cm from the pylorus for jejunum, and within 22-25 cm from the pylorus for ileum.

Physical appearance of the protein deprived intestine

There was a noticeable difference in the physical appearance of protein deprived gut compared to that of normal controls. The intestine was fragile, and appeared translucent. After long periods (e.g. 12 weeks), the gut was virtually transparent. The muscularis mucosa layer was much thinner and easier to remove from the gut samples. In general, the effects of protein deprivation on the physical appearance of intestine were more severe with increasing duration on the diet.

Structure of protein deprived and normal duodenum

Table 5.1 contains the measurements of villous height, crypt depth and crypt cell production rate (CCPR) made 2, 6 and 12 weeks after initiation of the diets.

Mean villous height of the 4% protein mice was significantly reduced at 2 and 6 weeks ($P < 0.01$), but was normal at 12 weeks ($P > 0.05$) compared to 24% protein control mice. At all time points examined, the crypt depth of deprived mice was decreased ($P < 0.01$). However, CCPR was decreased only in the 2 week protein deprived mice ($P < 0.05$ compared to controls).

There was no difference between the CCPR values for deprived and control mice at either 6 or 12 weeks ($P > 0.05$).

Structure of protein deprived and normal jejunum

A reasonably similar picture is observed when measurements of jejunum were taken from protein deprived and normal mice (Table 5.1). In this case, both villous height and crypt depth of 4% protein mice were significantly reduced at 2, 6 and 12 weeks compared to 24% protein mice ($P < 0.01$). In contrast, CCPR values of protein deprived mice were normal at all times examined ($P > 0.05$ compared to controls).

Structure of protein deprived and normal ileum

The results in Table 5.1 again demonstrate that protein deprivation for a period of 2, 6 or 12 weeks significantly reduced both villous height and crypt depth compared to that of protein sufficient control mice ($P < 0.01$). CCPR was significantly decreased in 2 week 4% protein mice ($P < 0.05$ compared to controls), but was normal in 6 and 12 week protein deprived mice ($P > 0.05$ compared to controls).

Disaccharidase activity of protein deprived and normal jejunum

Samples of jejunum were assayed for activity of four disaccharidase enzymes, lactase, sucrase, maltase and trehalase, and the results, expressed in μ.moles/minute/g. wet weight of tissue, are contained in Table 5.2.

There was no difference in lactase activity between 4% and 24% protein mice at any time point examined ($P > 0.10$).

However, sucrase activity in 4% protein mice was significantly raised at all these times (2, 6 weeks: $P < 0.01$, 12 weeks $P < 0.05$ compared to 24% protein mice). Similarly, maltase activity was increased in the protein deprived groups at 2 weeks ($P < 0.01$) and 6 weeks ($P < 0.05$), but was normal at 12 weeks ($P > 0.10$). There was no difference in trehalase activity between the two dietary groups at either 2 or 6 weeks ($P > 0.05$), but it was raised in 4% protein mice after 12 weeks ($P < 0.01$ compared to 12 week 24% protein mice).

Concluding remarks

The results contained in this chapter support the conclusion from other animal model studies that protein deprivation can induce marked changes in intestinal structure while the overall absorptive function of the gut can remain intact (Syme and Smith, 1982).

Both villous height and crypt depth are consistently decreased in protein deprived intestine at all times examined. De novo production of cells in the crypts is decreased after 2 weeks protein deprivation, but appears normal after prolonged periods. One exception to this is the CCPR value of 2 weeks protein deprived jejunum. Although the rate is decreased, this did not prove statistically different from normal jejunum at this time. It is interesting to note that while CCPR values for 24% protein mice decreased between week 2 and week 6 on this diet, the values for 4% protein mice increased. This pattern is also observed in the rat model

used by Syme (1982), and her proposal that protein deficiency inhibits the maturation of the intestine after weaning may be applicable here.

Activity of disaccharidase enzymes in the enterocyte brush border is used in this case as an indirect measure of the absorptive capacity of the intestine. Deficiency of one or all of these enzymes would result in malabsorption of the carbohydrate or carbohydrates in question. The data in Table 5.2 indicate that protein deprivation does not result in decreased levels of any of the enzymes tested. Indeed, sucrase, maltase and trehalase activities are all increased at different times in 4% protein animals. While it is possible that increased quantities of starch in the protein deprived diet could be responsible for the increased sucrase activity, it would be predicted that lactase would increase in parallel with this, as demonstrated by Koldovsky and his colleagues (Koldovsky, Bustamante, and Yamada, 1982). This does not occur in this model. The exact mechanism for the increase in enzyme levels, therefore, remains unclear.

Weeks from weaning	Diet	Duodenum			Jejunum			Ileum		
		V.H.	C.D.	CCPR	V.H.	C.D.	CCPR	V.H.	C.D.	CCPR
2	4%	646.6 ± 37.2	99.2 ± 2.5	7.2	577.4 ± 18.3	96.3 ± 3.2	7.1	429.2 ± 22.6	90.4 ± 2.7	6.1
	24%	786.4 ± 31.2	119.4 ± 5.2	11.1	763.2 ± 83.3	120.3 ± 3.0	10.8	632.2 ± 25.9	115.6 ± 2.0	12.3
6	4%	583.6 ± 28.6	95.1 ± 1.8	8.6	599.0 ± 48.2	98.9 ± 5.4	9.1	494.4 ± 50.9	92.6 ± 4.0	8.5
	24%	726.5 ± 26.5	116.9 ± 3.2	8.8	755.1 ± 42.1	124.1 ± 6.7	7.8	661.5 ± 27.7	113.9 ± 2.1	8.8
12	4%	715.6 ± 67.2	95.0 ± 3.0	8.9	633.4 ± 83.0	95.4 ± 7.4	10.9	320.2 ± 28.6	83.5 ± 5.6	6.9
	24%	789.6 ± 35.5	117.0 ± 3.9	10.1	779.0 ± 42.0	113.4 ± 3.3	10.6	448.1 ± 34.1	100.3 ± 3.0	10.6

Key

V.H. - villous height (μm)
C.D. - crypt depth (μm)
CCPR - crypt cell production rate (cells/hour)

Table 5.1: Measurements of intestinal structure in BDF_1 mice maintained on the 4% and 24% protein diets. Each group contained 6-8 mice and the figures represent the mean ± 1 s.e. Statistical comparisons between protein deprived and normal groups are given in the text.

Weeks from weaning	Diet	μ moles/minute/g. wet weight			
		Lactase	Sucrase	Maltase	Trehalase
2	4%	0.56 ±0.23	7.79 ±1.44	31.48 ± 2.77	9.19 ±1.79
	24%	0.68 ±0.26	3.24 ±1.38	18.72 ± 5.21	10.14 ±3.12
6	4%	1.18 ±0.23	8.00 ±2.53	22.25 ± 1.55	9.67 ±2.26
	24%	1.21 ±0.72	3.35 ±1.32	16.98 ± 3.38	9.71 ±3.40
12	4%	1.41 ±0.37	10.50 ± 2.26	26.37 ± 3.18	22.50 ± 3.14
	24%	1.96 ±0.60	6.94 ±1.64	26.19 ± 2.40	9.85 ±2.66

Table 5.2: Jejunal disaccharidase enzyme activity in BDF_1 mice maintained on the 4% or 24% protein diets. Each group contained 6–8 mice and the figures represent the mean ±1 s.d. Enzyme activity is expressed as μmoles/minute/g.wet weight of tissue. Statistical comparisons between protein deprived and normal groups are given in the text.

CHAPTER 6

THE EFFECT OF PROTEIN DEPRIVATION ON THE STRUCTURE OF IMMUNOLOGICAL ORGANS AND TISSUES

Introduction

The organs and tissues which comprise the immune system have proved to be very susceptible to the negative stimulus of protein deprivation (see Chapter 1). Cell depletion occurs in all structures examined, and appears most severe in the thymus, followed by the spleen and lymph nodes. Among the cells most affected are the non-recirculating T_1 cells, whereas recirculating, long-lived T_2 cells and resident B cells are least affected (Malavé et al., 1980). GALT structures e.g. mesenteric lymph nodes, have also been examined, and while they also show depletion, it is suggested that this is not as severe as observed in systemic tissues (Bell et al., 1976(a)). The aim of this chapter was to examine the organization of both systemic and mucosal lymphoid tissue in my model of protein deprivation. This would serve to highlight some of the similarities or differences which exist between my model and others. In addition, it would provide background information on the environment in which the induction of immune responsiveness or tolerance, examined in later chapters, would occur.

Experimental protocol

Groups of male and female BDF_1 mice aged 3 weeks were weaned onto diets containing 4% or 24% protein, and were maintained on these diets for a total of 2, 6 or 12 weeks. At these times, animals were sacrificed and samples of thymus, spleen, inguinal lymph node, mesenteric lymph node, Peyer's

patch, duodenum, jejunum, ileum and liver were removed. These organs and tissues were placed into fixative and taken for histological processing. In addition, samples of Peyer's patches were processed for examination by scanning electron microscopy.

Thymus

After 2 weeks protein deprivation, the thymus was much reduced in size and was difficult to find *in situ*. Microscopic examination of the normal thymus (Fig. 6.1(a)) revealed distinct separation between the inner medulla and the outer cortex, with both areas packed with thymocytes. The protein deprived thymus, however, (Fig. 6.1(b)) showed an ill-defined separation between medulla and cortex, with a vast reduction in the size and cellularity of the cortex. In contrast, this pattern was not repeated after longer periods (6 and 12 weeks) of protein deprivation (Fig. 6.1(c)). In this case, the thymus showed recovery of the cortico-medullary junction, with the cortex having a full complement of cells with no obvious areas of depletion.

Spleen

Macroscopically, this organ was reduced in size after all three periods of deprivation, compared to spleens from protein sufficient controls. Microscopically, the 2 week protein deprived spleen had recognizable red and white pulp regions, but showed a comparative loss of cellularity from

both. Figure 6.2(a) demonstrates the organization of a white pulp region from a normal mouse. Distinct lymphoid follicles, packed with cells and surrounded by a marginal sinus were present on either side of the central arteriole. The periarteriolar lymphatic sheath (PALS) was apparent as an aggregation of cells at the left hand side of the arteriole (arrowed on figure). In contrast, the fewer lymphoid follicles present in 2 week protein deprived spleen (Fig. 6.2(b)) were poorly defined, and contained few cells. The PALS in these mice seemed well maintained, although the organization around the arteriole was diffuse. After 6 weeks deprivation (Fig. 6.2(c)), the white pulp region appeared more cellular. However, although more follicles were observed, the marginal sinus around the follicles remained ill-defined. Furthermore, the PALS region around the arteriole appeared to contain few cells. This pattern was repeated with the 12 week protein deprived group.

Inguinal lymph node

Inguinal lymph nodes from protein deprived mice were surrounded by layers of fat and often proved exceptionally difficult to find *in situ*. The reduction in size was most severe after prolonged periods of deprivation. Protein restriction appeared to have little effect on the internal organization of lymph nodes. Although primary follicles were much smaller than in control nodes, cortical and medullary areas were well defined, with no obvious areas of cell depletion.

Mesenteric lymph node

This proved to be an essentially similar pattern to that observed with the inguinal lymph node. The nodes were difficult to find within layers of fat, and were reduced in size. Internal organization however, appeared normal, with all areas maintaining a normal complement of cells.

Peyer's patch

These lymphoid aggregates are found on the outer wall of the small intestine, and project into the lumen of the gut. Examination of the gut of protein deprived mice suggested that these animals had similar numbers of patches, but that they were smaller than those of control animals. Figure 6.3(a) shows the organization of a Peyer's patch from a 24% protein mouse. Two lymphoid follicles were observed. Both follicles had well defined germinal centres, surrounded by densely packed areas of small lymphocytes. Both the interfollicular and the dome areas of the patch also contained large numbers of cells. The patch from the 2 week 4% protein mouse however, appeared quite different (Fig. 6.3(b)). The germinal centre was smaller than in control mice, and was difficult to distinguish from the overlying corona of small lymphocytes. The dome region contained few cells, although the interfollicular area was not depleted. After 6 weeks protein deprivation (Fig. 6.3(c)), a large, clearly defined, germinal centre was again observed, and all areas of the patch were

packed with cells. It did appear, however, that the cap of small lymphocytes over the germinal centre was smaller than in controls. This pattern of restored organization of the patch was again apparent in 12 week protein deprived animals.

S.E.M. examination of protein deprived and normal mice suggested a difference in the degree of projection of the patch into the gut lumen between the two groups. Figure 6.4 is a scanning electron micrograph of a Peyer's patch from a 24% protein mouse. The domes of the patch were quite easily seen amidst the surrounding villi. In contrast, in a similar region from a 4% protein mouse, the domes were barely visible (Fig. 6.5). Examination of the surface of the patch revealed a number of M cells (arrowed in Fig. 6.6), which act as specialized vehicles for uptake of antigen (Owen, 1977). Quantitatively, there appeared to be no gross difference in numbers of these cells between 4% and 24% protein mice.

IEL counts in duodenum, jejunum and ileum

Table 6.1 contains the counts of IELs from duodenum, jejunum and ileum of protein deprived and normal mice. After 2 weeks deprivation, IEL numbers were normal in duodenum ($P > 0.05$) but were increased in jejunum ($P < 0.05$) and ileum ($P < 0.01$) compared to controls. With increasing length of restriction however (6 weeks), levels of IELs were decreased in duodenum ($P < 0.05$), jejunum and ileum ($P < 0.01$). After the 12 weeks period, only jejunum was taken from the mice, and 4% protein mice were shown to have lower numbers of IELs than 24% protein control mice ($P < 0.01$).

Liver

This organ was the least affected by protein deprivation of all those examined in this section. The liver of protein deprived mice was reduced in size proportionate to the reduction in overall body size, and it appeared normal in colour. Internal organization of protein deprived liver was similar to that of control mice, however, after long periods of deprivation (6 weeks and 12 weeks), there was a reduction in the density of the surrounding trabeculae.

Concluding remarks

The results contained in this chapter confirm and extend the finding from other animal model studies that feeding weanling mice a protein deprived diet can result in a loss of lymphoid tissue which is proportionately greater than the loss of body weight. This is most severe in the thymus, followed by the spleen, inguinal and mesenteric lymph nodes, with very little effect observed in the liver. In addition, other GALT structures which were examined also show cellular depletion.

Several points arising from these studies are of particular interest. First, internal organization and cellularity of the thymus (and to a lesser extent the spleen and Peyer's patches) is more severely affected after 2 weeks deprivation, than after 6 weeks deprivation. This recovery effect has not previously been reported from other animal model studies, and may be a characteristic of the strain of mouse used in

this work. Second, the PALS region of protein deprived mice appears unaffected by 2 weeks deprivation, but with continuing periods, is depleted of small lymphocytes. Third, IEL counts in jejunum and ileum are increased after 2 weeks deprivation, but by 6 and 12 weeks are reduced below control levels. This disparate effect of protein deprivation on IEL numbers after different times will be considered later.

Weeks from weaning	Diet	Duodenum	Jejunum	Ileum
2	4%	7.7 ± 1.0	8.5 ± 1.0	9.5 ± 1.3
	24%	7.7 ± 0.7	6.8 ± 0.8	6.9 ± 1.1
6	4%	13.2 ± 2.1	14.3 ± 1.1	11.1 ± 1.1
	24%	17.5 ± 2.5	17.9 ± 0.9	14.8 ± 0.8
12	4%	N.D.	12.4 ± 2.4	N.D.
	24%	N.D.	16.4 ± 1.2	N.D.

N.D. - not determined

Table 6.1: IEL counts in BDF_1 mice maintained on the 4% or 24% protein diets. Samples of duodenum, jejunum and ileum removed from the gut as stated (Chapter 5). Counts expressed as IEL number per 100 epithelial cells, and figures represent the mean ± 1 s.d. of 6-8 mice. Statistical comparisons between protein deprived and normal groups are given in the text.

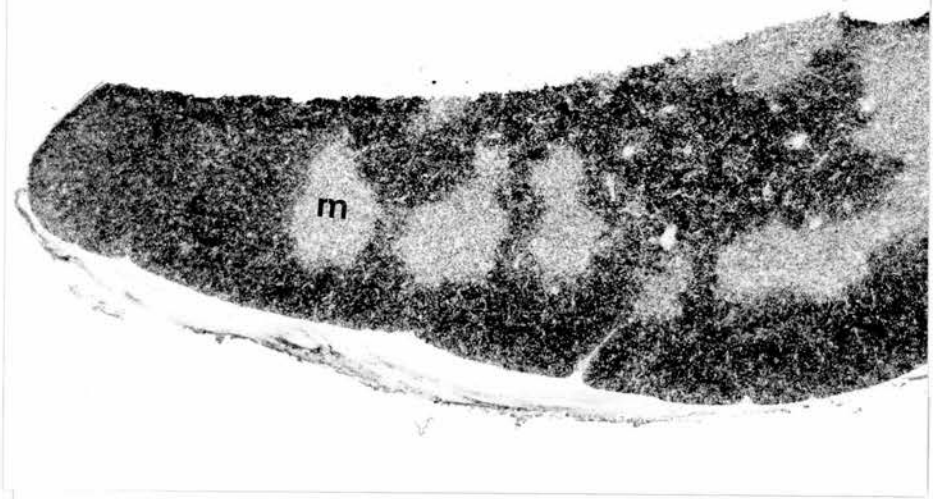

Fig. 6.1(a):
24% protein for
2 weeks (H & E
magnification x 3

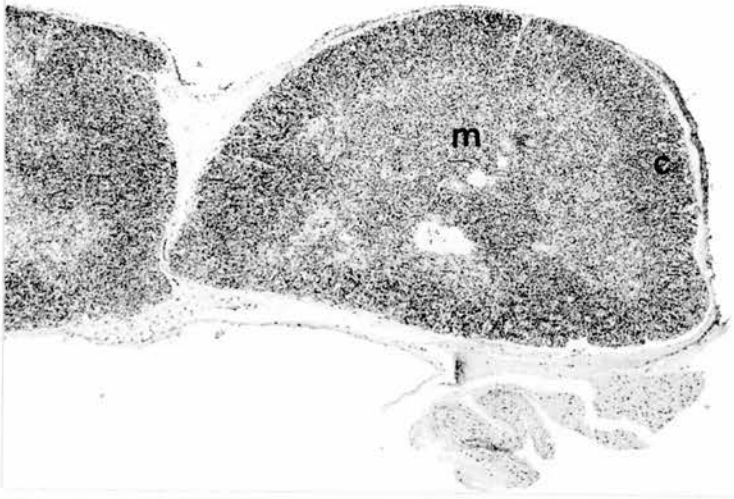

Fig. 6.1(b):
4% protein for
2 weeks (H & E
magnification x 5

Fig. 6.1(c):
4% protein for
6 weeks (H & E
magnification x 35

Fig. 6.1: Thymus of BDF_1 mice maintained on 4% or 24% protein diets from weaning. Medulla (M) and cortex (C) are marked on plates.

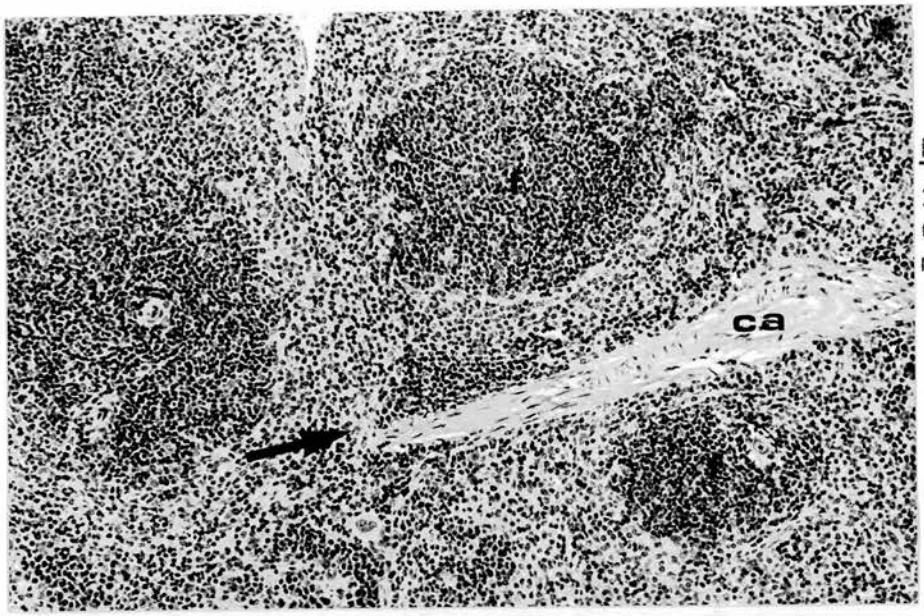

Fig. 6.2(a):
24% protein for 6 weeks (H & E magnification × 125)

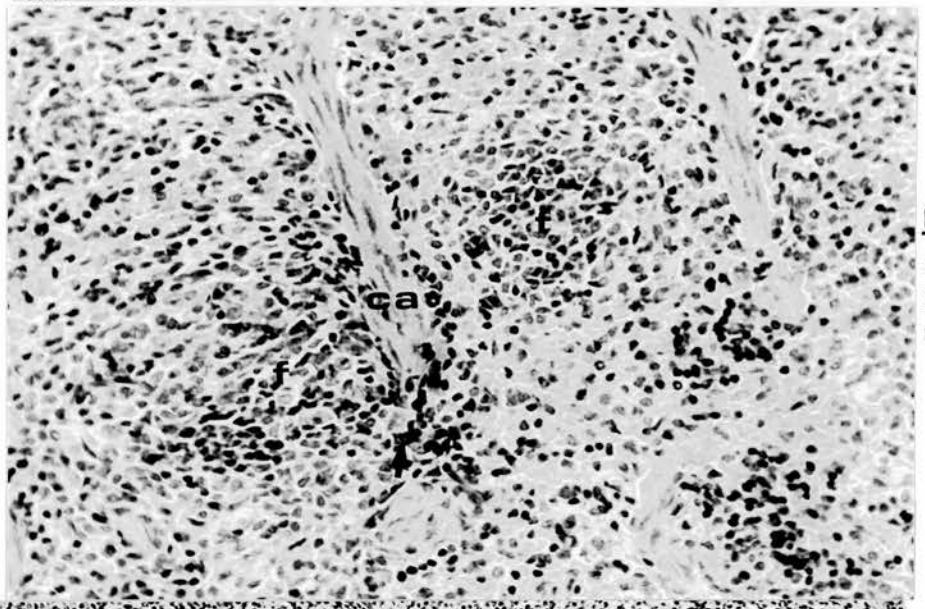

Fig. 6.2(b):
4% protein for 2 weeks (H & E magnification × 320)

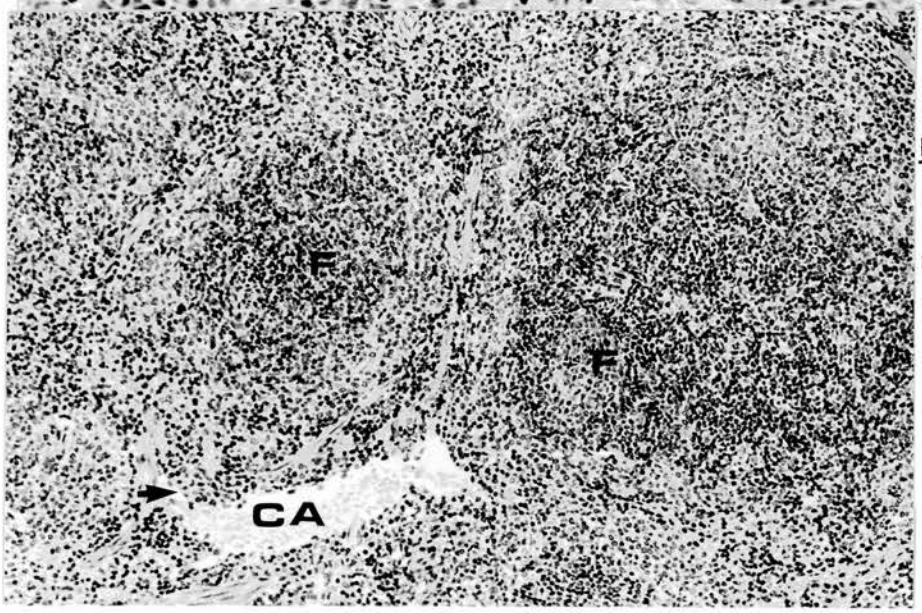

Fig. 6.2(c):
4% protein for 6 weeks (H & E magnification × 125)

Fig. 6.2: Spleen of BDF_1 mice maintained on 4% or 24% protein diets from weaning. Lymphoid follicles (F) surrounding the central arteriole (CA) are marked on plates. Periarteriolar lymphatic sheath (PALS) regions are arrowed on plates for identification.

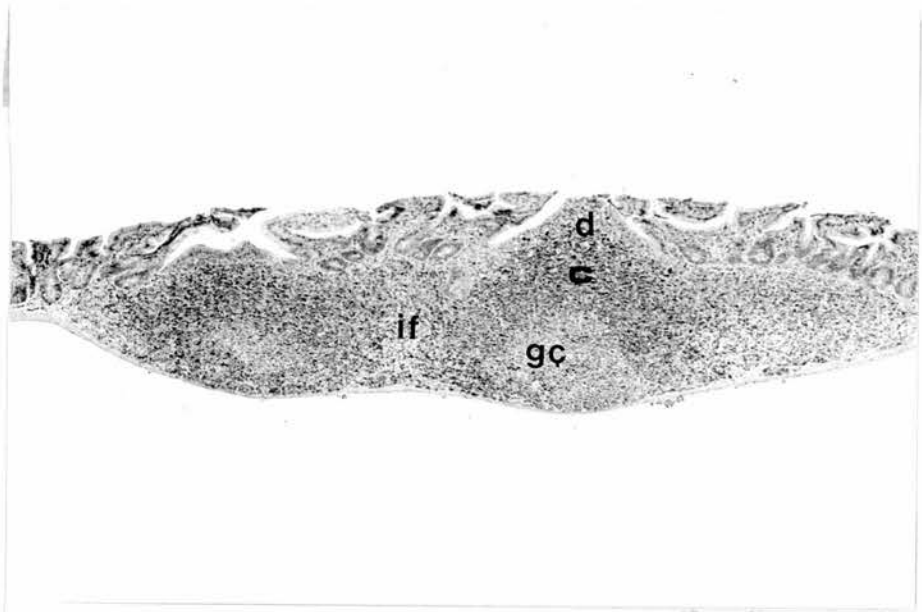

Fig. 6.3(a):
24% protein for 2 weeks (H & E magnification x 50)

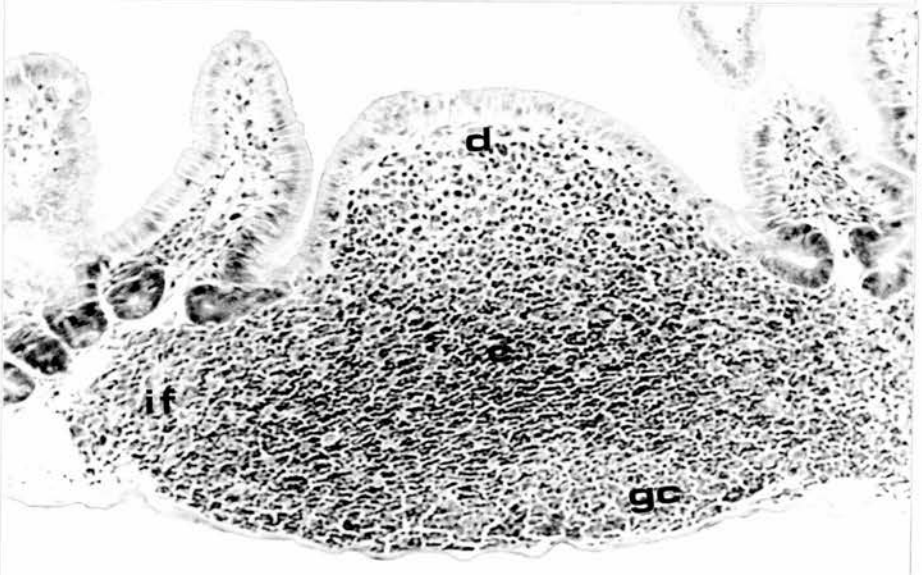

Fig. 6.3(b):
4% protein for 2 weeks (H & E magnification x 160)

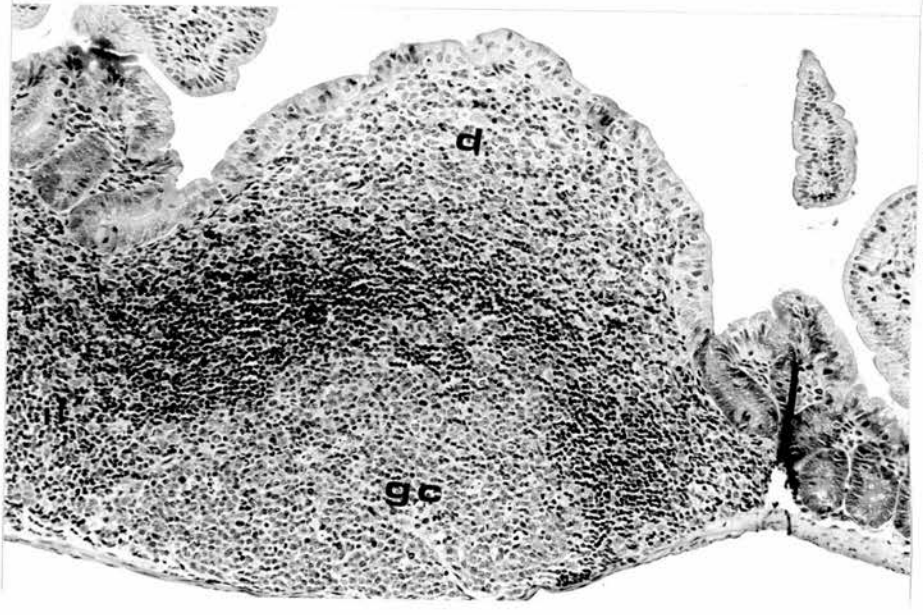

Fig. 6.3(c):
4% protein for 6 weeks (H & E magnification x 125)

Fig. 6.3: Peyer's patches of BDF_1 mice maintained on 4% or 24% protein diets from weaning. Regions within the patch indicated are:- the dome region (D), cap or corona of small lymphocytes (C), germinal centre (GC) and the interfollicular area (IF).

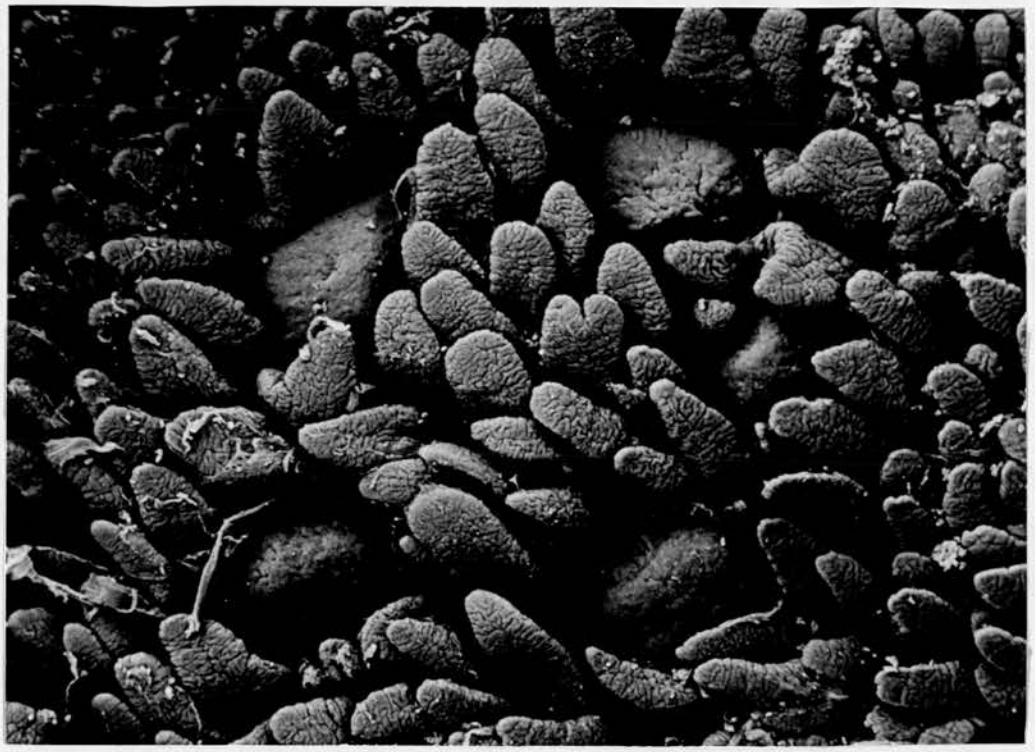

Fig. 6.4: Scanning E.M. appearance of the lumenal surface of a Peyer's patch from a 24% protein mouse. Note the projections of the patch into the gut lumen, with surrounding villi (mag. x 52).

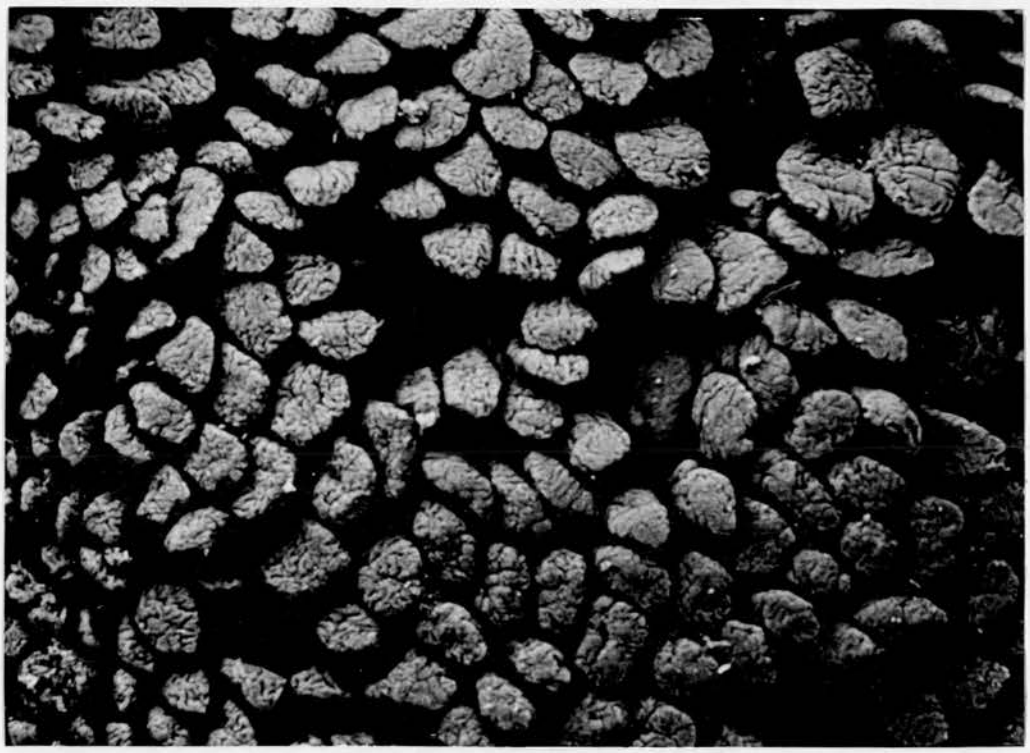

Fig. 6.5: Scanning E.M. appearance of the lumenal surface of a Peyer's patch from a 4% protein mouse. By contrast, the domes of the patch are partially hidden (mag. x 57).

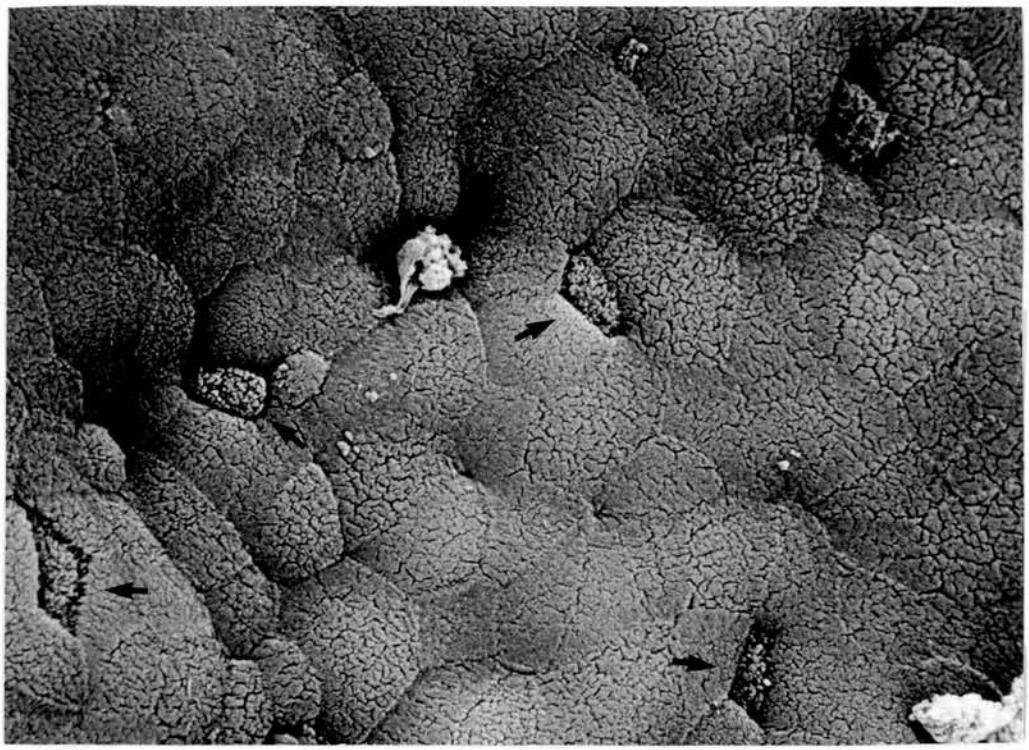

Fig. 6.6: Scanning E.M. of the dome epithelium of a 24% protein Peyer's patch. M cells (arrowed on plate) are clearly distinguishable from the surrounding enterocytes (mag. × 2050).

CHAPTER 7

THE EFFECT OF PROTEIN DEPRIVATION ON T CELL FUNCTION

Introduction to experiments

Animal models of protein deprivation have shown that the thymus-dependent limb of immunity may be particularly impaired (data reviewed in Chapter 1). The aim of the experiments detailed in this chapter was to examine T cell function in the animal model which is used throughout this thesis.

The following approaches were used to assess T cell function:-

a) Primary antibody response to the thymus-dependent antigen OVA

b) DTH response to OVA

c) Passive transfer of DTH to naive recipients

d) Lymphocyte transformation using the mitogen PHA

e) The ability to induce a GvHR in F_1 animals using parental cells.

These tests measured, either directly or indirectly, the immune competence of T lymphocytes from the malnourished host.

A. PRIMARY ANTIBODY RESPONSE TO OVA

Experimental protocol

Female BDF_1 and BALB/c mice aged 3 weeks were weaned onto the 4% or 24% protein diets, and were maintained on them for 3 weeks (BDF_1 and BALB/c) or 11 weeks (BDF_1 only) before immunization with 100 μg OVA/CFA i.d. into one rear footpad. The same diets as before were continued, and the

animals were bled 14 and 21 days after immunization. To minimize animal costs, the mice used in this experiment were also used in the control saline-fed group in the first oral tolerance experiment. 4-8 mice were used per group, and antibody levels were assessed by isotype-specific ELISA.

Antibody responses

The results in Figure 7.1(a) show that after short-term protein deprivation (3 weeks before immunization), levels of IgM antibodies in BDF_1 mice were increased at day 21, but not at day 14, when compared to the 24% protein mice ($P < 0.05$). In contrast, IgG levels were significantly decreased in protein deprived mice at both day 14 and day 21 ($P < 0.01$). However when BDF_1 mice were protein deprived for 11 weeks before immunization (Fig. 7.1(b)), there were no differences in the IgM or IgG responses between 4% protein or 24% protein mice at either day examined ($P > 0.05$).

In contrast to BDF_1 mice, the results in Figure 7.2 illustrate that short term protein deprived BALB/c mice produced similar levels of IgM and IgG in response to OVA as did 24% protein mice at both day 14 and day 21 ($P > 0.05$).

B. DTH RESPONSE TO OVA

Experimental protocol

The same groups of mice which were bled in Section A for antibody levels, were also used to assess DTH responsiveness. Twenty one days after immunization, animals were

challenged with 100 μg OVA/SAL into the contralateral footpad. The footpad was measured immediately before, and 24 hours after challenge and the difference in thickness expressed in mm.

DTH responses

The results in Figure 7.3 indicate that short term protein deprivation impaired the ability to mount a DTH response upon challenge with antigen ($P < 0.01$ compared to 24% protein mice). However, after longer periods of deprivation, in this case 11 weeks before immunization, DTH responsiveness to OVA was restored ($P > 0.05$ compared to 24% protein mice).

The adverse effect which short term protein deprivation had for DTH responses was not confined to BDF_1 mice. BALB/c mice, protein deprived for 3 weeks before immunization, also had an impaired DTH response to OVA ($P < 0.01$ compared to 24% protein mice).

C. LOCAL PASSIVE TRANSFER OF DTH

The cellular events which participate in the DTH response are summarized in Figure 7.4. A defect in any or all of these limbs might account for the depressed DTH response observed in short term protein deprived mice. The aim of this section was to examine which limb or limbs were affected in these mice, by passive transfer of primed cells plus antigen to naive recipient footpads to attempt to elicit a

response. Transfer of primed cells from deprived mice to normal footpads would examine the initial priming of the T_{DTH} cell, while transfer of primed cells from normal mice to protein deprived footpads would examine the inflammatory limb of the response in these mice.

Experimental protocol

It was intended to examine if protein deprived mice were able to generate T effector cells for DTH responses by transferring cells from these mice into the footpads of protein sufficient recipients with antigen. However, the very low cell yield per lymph node in low protein animals ($6-9 \times 10^5$ viable cells/node compared to $7-12 \times 10^6$ viable cells/node of normal animals) meant that insufficient numbers of mice were available to act as cell donors. Therefore, all cell donors in this experiment were 24% protein maintained.

Female BDF_1 mice were placed onto the 4% or 24% protein diets at weaning, and 3 weeks later, were immunized with 100 µg OVA/CFA i.d. into both rear footpads. Control animals received 0.05 ml SAL/CFA. The inguinal and popliteal lymph nodes were removed 21 days after immunization, and 10^7 cells plus 100 µg OVA were transferred into the footpad of a naive syngeneic recipient. The recipient animals had been maintained on the diets for a minimum of 3 weeks and a maximum of 5 weeks from weaning. The footpads were measured immediately before and 24 hours after transfer of cells plus antigen, and the difference expressed in mm. Each group contained 6-8 mice.

DTH responses

Figure 7.5 shows that OVA primed cells plus OVA gave a positive DTH response when transferred into the footpad of a 24% protein recipient, but gave no response when transferred to the footpad of a 4% protein recipient ($P < 0.01$). SAL primed cells were unable to give a positive response when transferred into a 24% protein footpad with OVA ($P < 0.01$ compared to OVA primed cells plus OVA).

D. MITOGEN STIMULATION OF PROTEIN DEPRIVED LYMPHOID CELLS

The transformation of lymphoid cells following stimulation with mitogens provides a test to measure the basic reactivity of these cells. Much of the evidence concerning the effect of protein deprivation on mitogen responsiveness is conflicting (see Chapter 1). The aim of this section was to investigate the effect of long term protein deprivation on the response of lymphocytes to mitogen, in the model which I was using.

Experimental protocol

Female BDF_1 mice were maintained on the 4% or 24% protein diets for 12 weeks from weaning. Popliteal and inguinal lymph nodes were removed and the lymphoid cells were stimulated with the mitogen PHA. Control samples received no PHA.

Results

The results in Table 7.1 indicate that when the response is assessed by the c.p.m. data, transformation to PHA was decreased in protein deprived mice compared to 24% protein controls ($P < 0.05$). However, on the basis of S.I. data, responsiveness of lymphocytes to PHA was increased in the deprived mice.

These results suggest that one reason for the conflicting reports mentioned previously may lie with the method of data presentation.

E. GvHR IN F_1 MICE USING PROTEIN DEPRIVED LYMPHOCYTES

In order to investigate the effect of protein deprivation on the function of lymphoid cell populations, there is a requirement to remove the cells from the restrictive environment of the protein deprived host and examine them either *in vitro* or *in vivo* within normally nourished recipients. The GvHR induced in F_1 hosts by injection of parental lymphocytes offers such a test. The severity of the reaction is assessed by measuring the increase in weight of the F_1 spleen, which is a T cell mediated phenomenon (Grebe and Streilein, 1976). Additionally, the changes occurring in the gut mucosa at this time are caused by a local CMI reaction (Mowat and Ferguson, 1981(b)). It was intended, therefore, to examine the function of protein deprived lymphoid cells by investigating their ability to produce the splenic and intestinal changes associated with a mild GvHR.

Experimental protocol

Parental strain female mice (C57BL/6J: H-$2^{b/b}$) were maintained on the 4% or 24% protein diet for 2 weeks from weaning, and were then sacrificed to serve as spleen cell donors. Recipient BDF_1 mice (aged 6-8 weeks), which had been maintained on the normal protein-sufficient laboratory diet from weaning each received 4.5×10^7 cells i.p. from either 4% or 24% protein donors. Control animals received 0.2 ml RPMI 1640 alone. Fourteen days after induction of GvHR, all animals were killed, their spleens were removed and weighed, and samples of jejunum removed for histological and microdissection analysis.

Cell yields

4% protein donors ⟶ 1.5×10^7 viable cells/spleen

24% protein donors ⟶ 8×10^7 viable cells/spleen

Spleen index

The relative spleen weights and spleen indices are given in Table 7.2. Both 4% and 24% protein parental cells induced significant splenomegaly in F_1 recipients ($P < 0.01$ compared to control spleen). The difference in splenic indices, however, did not reflect a significant difference between the relative spleen weights of the GvHR groups ($P > 0.05$).

Mucosal morphology

The results in Table 7.2 indicate that the numbers of IELs in the GvHR groups were not significantly increased over control values ($P > 0.05$) in this experiment. However, there was a significant difference in counts between the two donor groups ($P < 0.01$).

Microdissection analysis of the jejunal samples revealed that both parental cell inocula caused significant increases in crypt depth ($P < 0.01$ compared to control value), but no difference in villous height ($P > 0.05$ compared to control value). CCPR values from both GvHR groups were slightly increased over the control value, but these did not prove to be significant ($P > 0.05$).

F. CONCLUDING REMARKS

The results indicate that short term protein deprivation altered the antibody response to the thymus-dependent antigen OVA by producing proportionately more IgM and less IgG antibody than was produced by normally nourished control mice. This effect did not occur when a longer period of deprivation was induced, nor when mice of a different inbred strain were subjected to protein restriction. The response after short term deprivation is phenotypically immature, and the switch of antibody isotypes from IgM $\rightarrow$ IgG during the course of the primary response, appears to have been prevented.

The DTH response was similarly impaired after short term protein deprivation, but was restored after longer periods. Passive transfer of the response revealed that the inflammatory limb of DTH was restricted in protein deprived mice.

Finally, injection of protein deprived parental cells into F_1 recipients to initiate a mild GvHR indicated that the T cell function of these mice was normal.

In summary, T cell function, when assessed in in vivo tests, appeared depressed. However, when the cells were removed from the malnourished host, their function was normal. These findings are similar to those obtained from other animal models of protein deprivation (Chapter 1). Although the functions of T cells in immune responses are multiple and diverse, it is clear that additional factors such as impaired migration and proliferation within a restrictive environment may also contribute to the depressed responses of protein deprived animals.

Diet	4% (c.p.m.)	24% (c.p.m.)
PHA + cells	3190 ± 1561	5374 ± 989
cells only	230 ± 59	598 ± 105
S.I.	13.9	9.0

Table 7.1: PHA stimulation of LN cells from BDF_1 mice maintained on the 4% or 24% protein diets for 12 weeks from weaning. Control cells received no PHA. Figures represent the mean ± 1 s.d. of 6-8 mice and the comparisons between the groups are detailed in the text. S.I. calculated as described in Chapter 3.

Cells received	Spleen wt. (mg/10g body weight)	Spleen index	IEL (per 100 e.c.)	Villous height (μm)	Crypt Depth (μm)	CCPR (per hr.)
24% C57BL/6J	76.0 ± 21.2	2.15	18.5± 2.1	785.5 ± 34.5	132.4 ± 4.0	11.5
4% C57BL/6J	58.6 ± 16.8	1.66	14.4±1.5	800.3 ±27.0	135.6 ± 3.7	12.0
Control (0.2 ml RPMI)	35.3 ± 3.7	-	16.3±1.9	777.1 ±60.9	115.7 ± 1.9	9.8

Table 7.2: GvHR induced in BDF_1 mice by injection of 4% or 24% protein parental cells. Control group received 0.2 ml RPMI i.p. The figures represent the mean ± 1 s.d. of 6-8 mice, and comparisons between the GvHR and control groups are given in the text. Spleen index calculated as described in Chapter 3.

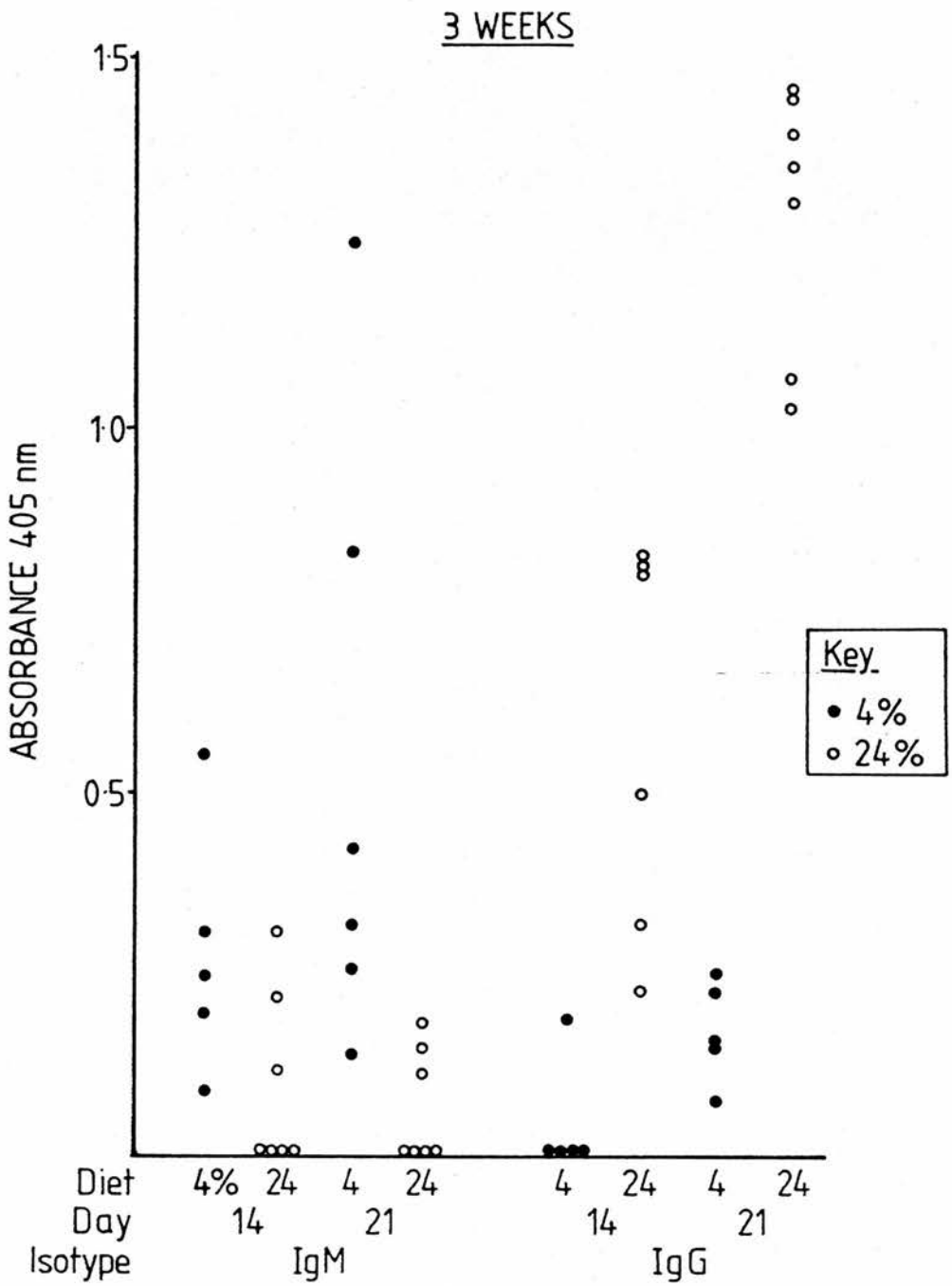

Fig. 7.1(a): Primary IgM and IgG antibody responses of BDF_1 mice maintained on the 4% or 24% protein diet for 3 weeks from weaning before OVA immunization. Animals were bled at d.14 and 21. Statistical comparisons between the groups are given in the text.

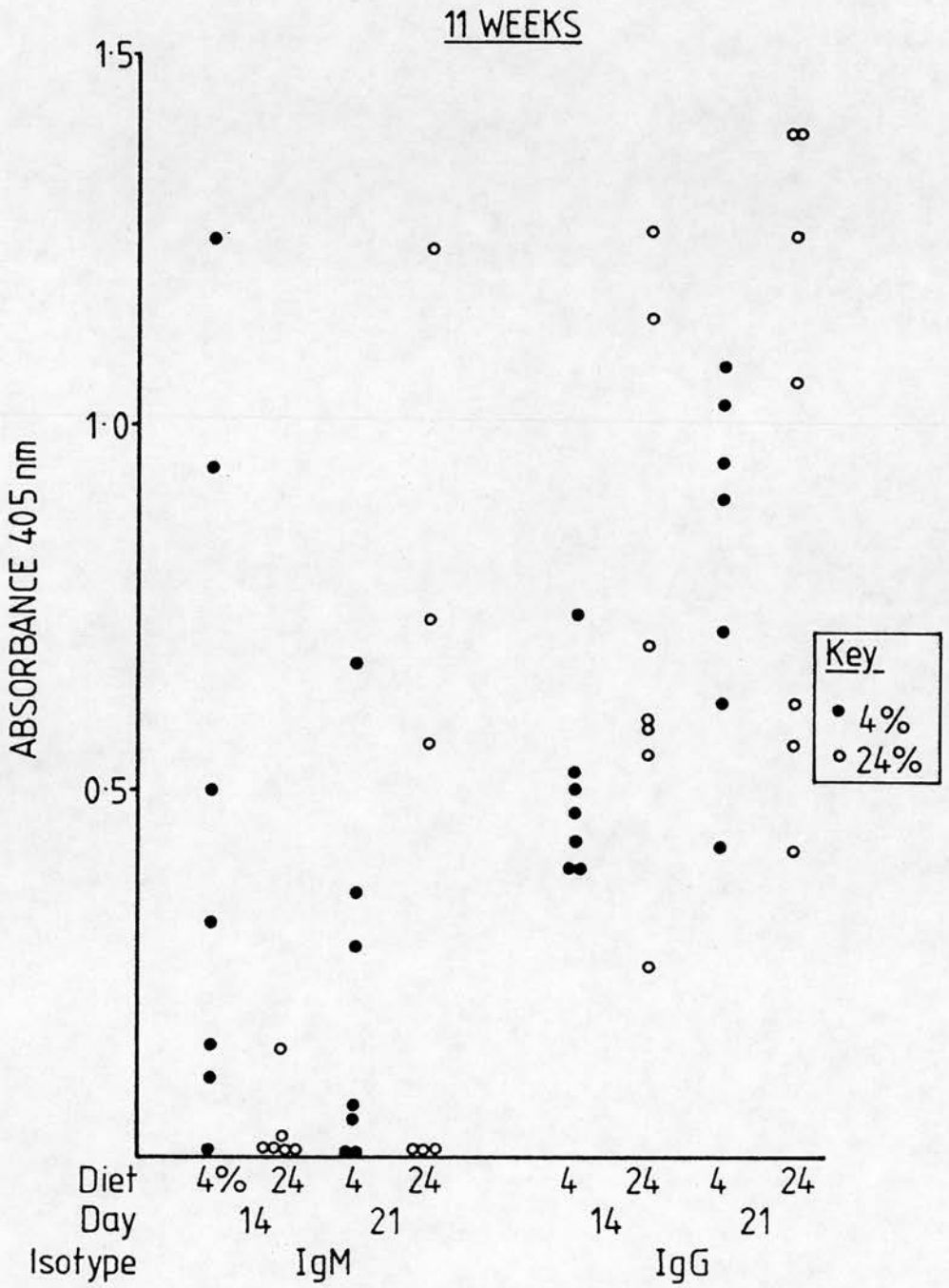

Fig. 7.1(b): Primary IgM and IgG antibody responses of BDF_1 mice maintained on the 4% or 24% protein diet for 11 weeks from weaning before OVA immunization. Animals were bled at d.14 and 21. Statistical comparisons between the groups are given in the text.

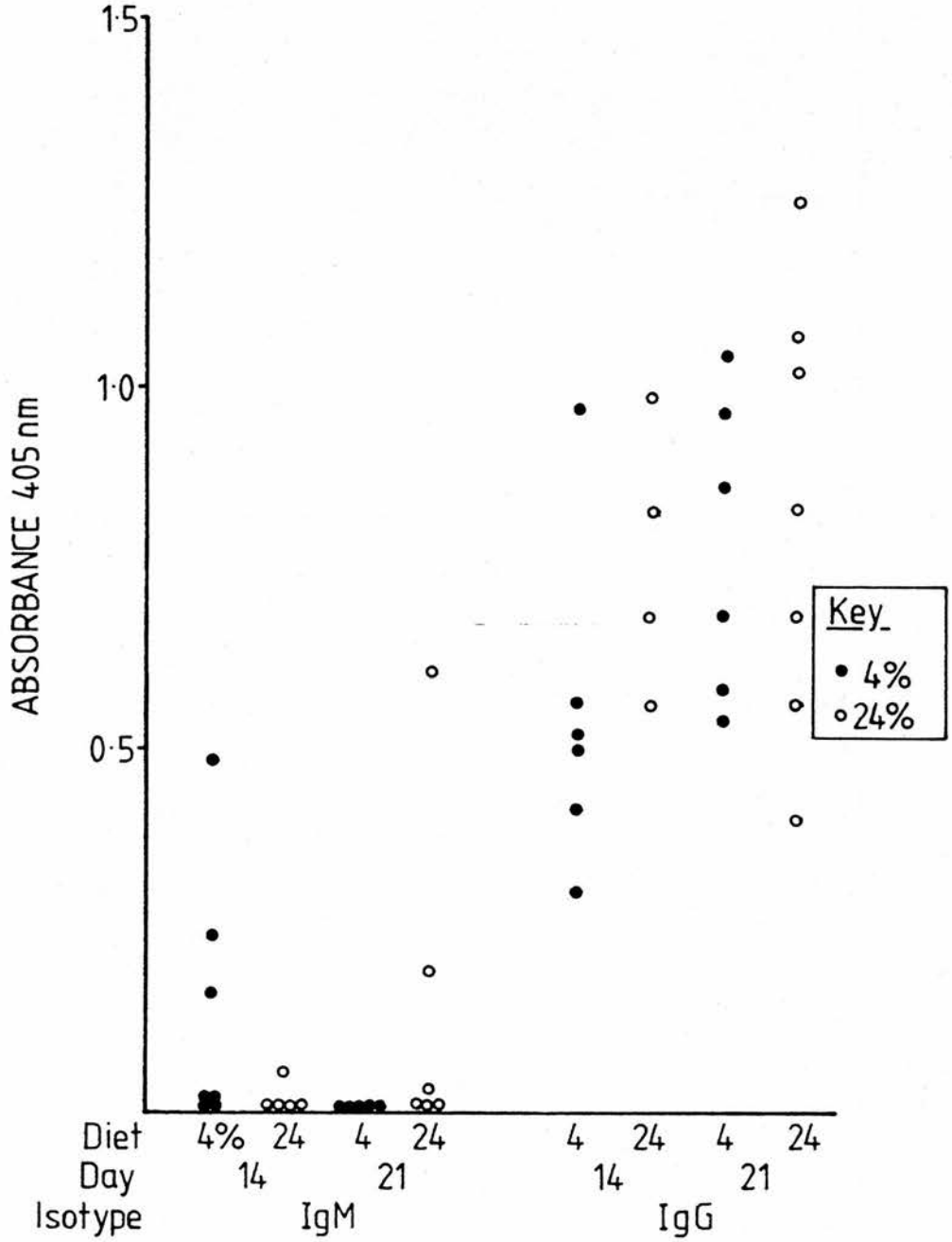

Fig. 7.2: Primary IgM and IgG antibody responses of BALB/c mice maintained on the 4% or 24% protein diet for 3 weeks from weaning before OVA immunization. Animals were bled at d.14 and 21. Statistical comparisons between the groups are given in the text.

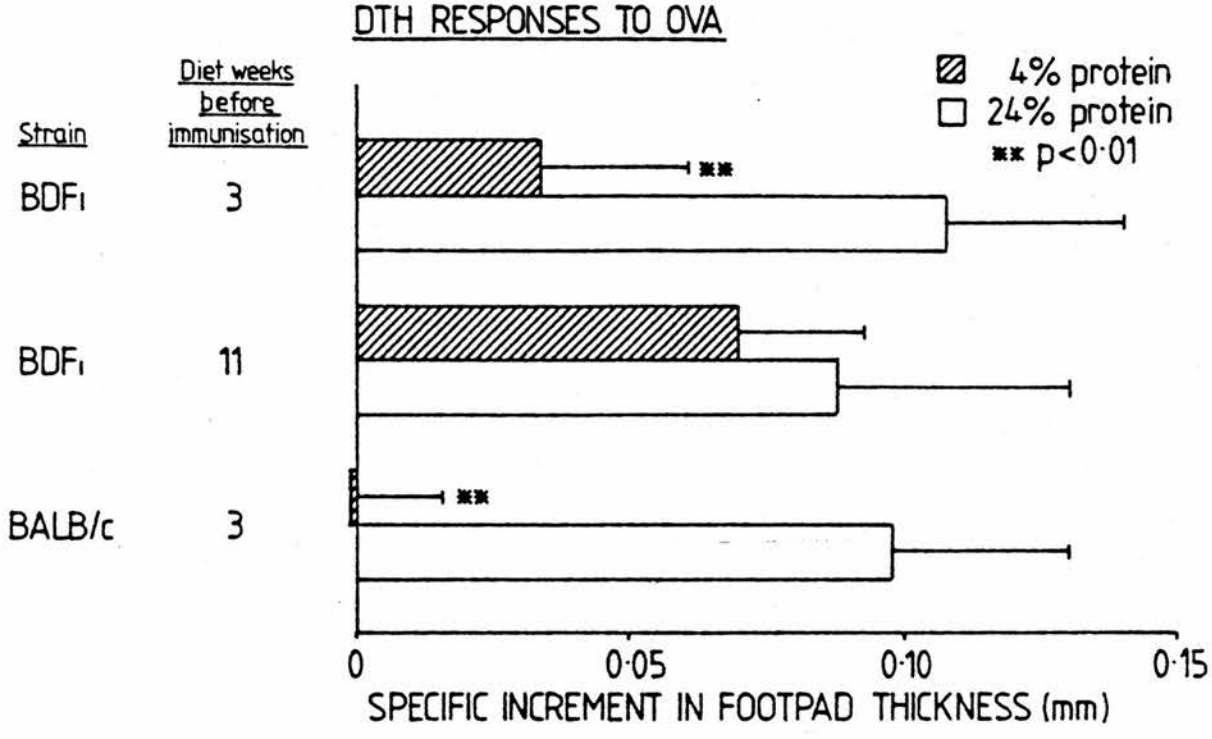

Fig. 7.3: DTH responses to OVA in BDF_1 and BALB/c mice. The mice were maintained on the 4% or 24% protein diet for 3 or 11 weeks before immunization, and DTH was assessed 21 days later. The bars represent the mean + 1 s.d. of 4-8 mice. The responses of the protein deprived groups are compared to the 24% protein controls.

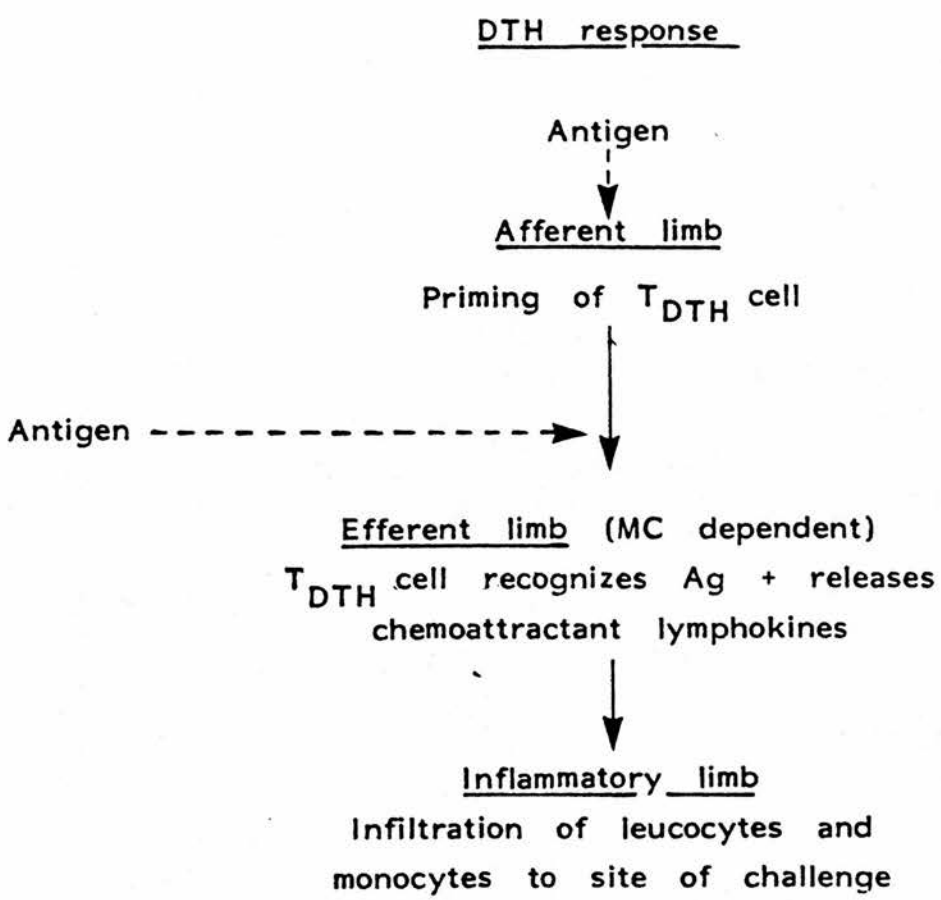

Fig. 7.4: Stages involved in the DTH response to antigen.

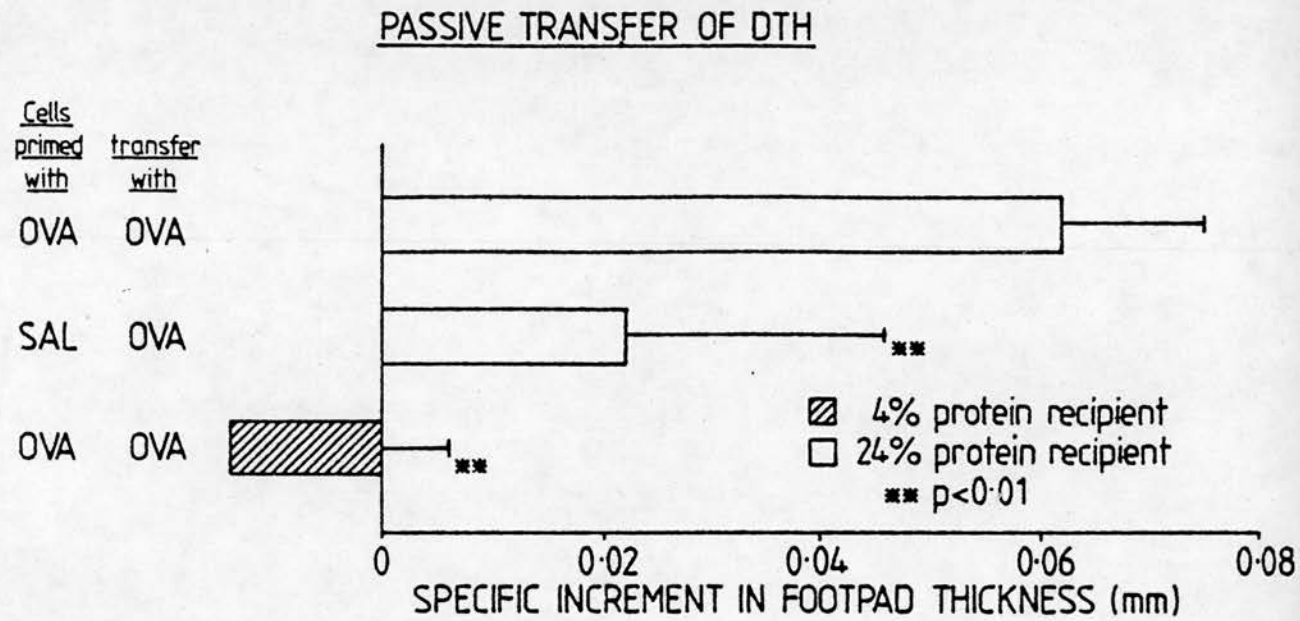

Fig. 7.5: Local passive transfer of DTH. 10^7 primed LN cells were transferred to the footpads of naive 4% or 24% protein recipients plus OVA. Control animals received 10^7 SAL primed cells. The response was measured 24 hrs after transfer and the bars represent the mean + 1 s.d. of 6-8 mice. The responses of the control animals and 4% protein recipients are compared to the response of the 24% protein recipients of OVA primed cells plus OVA.

CHAPTER 8

THE EFFECT OF PROTEIN DEPRIVATION ON ORAL TOLERANCE FOR HUMORAL AND CELL MEDIATED IMMUNE RESPONSES

Introduction to experiments

Of the several mechanisms which are implicated in the control of oral tolerance, T_s cells have been amongst the most widely studied. In conditions where these cells are deficient, the state of tolerance may be impaired (see Chapter 2). Reports from several models of protein deprivation indicate that this is one condition in which activity of T_s cells may be preferentially reduced (see Chapter 1). This suggests that tolerance will be impaired in protein deprived animals. The aim of this chapter, therefore, was to examine the effect of protein deprivation on the phenomenon of oral tolerance induced by a single feed of antigen.

General experimental protocol

The protocol used for all oral tolerance experiments is shown in Figure 8.1. Female BDF_1 or BALB/c mice aged 3 weeks were used in all experiments, and each experimental group contained 4-8 mice. In nutritional rehabilitation experiments, refeeding was started at d.-7 immediately after oral administration of antigen. Additionally, in some experiments, animals were bled at d.0, prior to immunization, to check for antibodies induced by feeding antigen.

A. THE EFFECT OF CONTINUOUS PROTEIN DEPRIVATION ON ORAL TOLERANCE TO OVA IN BDF_1 MICE

Experimental protocol

Groups of female BDF_1 mice were maintained on the diets

for 2 or 10 weeks from weaning before receiving a feed of antigen (d.-7: Fig. 8.1), and were continued on the same diet for a further 4 weeks until the end of the experiment. Animals were immunized 1 week after oral administration of antigen.

Antibody responses

Figure 8.2 shows the antibody responses of mice maintained on the diets for 2 weeks before feeding antigen. No antibodies were detected prior to immunization (d.0) in any group (data not shown). SAL immunized animals of both dietary groups showed small amounts of antibody at both d.14 and 21. 4% protein SAL fed OVA immunized mice had significantly less antibody at both d.14 and 21 compared to the equivalent 24% protein group ($P < 0.05$). Feeding OVA before immunization significantly reduced antibody titres in both 4% and 24% protein groups at d.14 and d.21 ($P < 0.01$ compared to the appropriate SAL fed OVA immunized group). In addition, 4% protein OVA fed mice had decreased antibody titres compared to 24% protein OVA fed mice at both days ($P < 0.01$). Tolerance was OVA specific, as both 4% and 24% protein BSA fed groups had normal antibody responses at d.14 and d.21 when compared to their corresponding SAL fed control group ($P > 0.05$).

Figure 8.3 shows the antibody responses of mice maintained on the diets for 10 weeks before feeding antigen. No antibodies were detected prior to immunization (d.0) in

any group (data not shown). In this case, 4% protein SAL fed OVA immunized mice had normal antibody titres at d.14 and d.21 compared to 24% protein mice ($P > 0.05$). 24% protein mice were not tolerized by feeding OVA ($P > 0.05$ compared to SAL fed group), however the response of 4% protein mice was suppressed by feeding ($P < 0.05$ at d.14, $P < 0.01$ at d.21 compared to SAL fed group). The tolerance was OVA specific as the 4% protein HSA fed group had a normal antibody response at days 14 and 21 compared to the SAL fed control group ($P > 0.05$).

DTH responses

The results in Figure 8.4 illustrate that short term protein deprived mice had a decreased DTH response to OVA compared to the 24% protein control mice ($P < 0.01$). Feeding OVA to 24% protein mice resulted in 86% suppression ($P < 0.01$) of their response. As there was no positive response in the 4% protein group, it was impossible to assess the affect of feeding OVA prior to immunization in these mice. 24% protein, BSA fed mice had a response midway between the positive control group and the tolerant group, and was significantly different from both ($P < 0.05$).

Figure 8.5 shows the DTH responses of mice maintained on the diets for 10 weeks before feeding antigen. The response of 24% protein mice was 78% suppressed after feeding OVA ($P < 0.01$ compared to SAL fed mice). 4% protein SAL

fed mice had a restored ability to mount a positive DTH response and it was shown that feeding OVA to 4% protein mice resulted in only 47% suppression ($P < 0.05$) of the response. Feeding HSA prior to immunization in 4% protein mice resulted in suppression of the DTH response ($P < 0.01$), however, no suppression was observed in the 24% protein HSA fed group. The reason for this anomaly is not known.

Comments

These experiments have demonstrated that continuous protein deprivation, initiated at weaning, can profoundly alter the state of tolerance for antibody and DTH responses, induced by a single feed of OVA.

After short term protein deprivation, feeding the antigen to 4% protein mice prior to immunization resulted in very profound suppression of the antibody response compared to that observed with protein sufficient mice. With longer periods of deprivation, the 4% protein mice showed significant suppression after feeding. In contrast, the 24% protein mice were unable to be tolerized for subsequent humoral responses by this method.

The DTH response was depressed by short term protein deprivation but was restored after longer periods. Therefore, the effect of protein deprivation on oral tolerance for DTH could be assessed only in those mice which had been maintained on the diet for 10 weeks from weaning. The degree of suppression induced in these mice by feeding antigen was

decreased compared to that in 24% protein mice.

The findings indicate that protein deprivation has a disparate effect on orally induced tolerance for antibody and DTH responses. Furthermore, it is likely that different control mechanisms are responsible for the tolerance of humoral and cell mediated responses.

B. ISOTYPE SPECIFIC ELISAs FOR THE DETECTION OF μ-CHAIN AND γ-CHAIN ANTIBODIES

The ELISA method used in the previous section measured only IgG antibodies. The finding that protein deprived mice had more profound oral tolerance for antibody responses applied therefore only to this isotype. As protein deprivation can increase the proportion of IgM produced in response to antigen (see Chapter 1), it was necessary at this stage to develop the ELISA method to measure both isotypes. This method was used in the experiments described in Chapter 7, and in all subsequent experiments.

Experimental protocol

The sera which were tested in Section A for antibodies to OVA, were retested using the new methods.

Antibody responses

I. IgM

Figure 8.6 shows the IgM anti-OVA antibody response in animals maintained on the diets for 2 weeks before feeding.

The titres of IgM produced varied considerably within each group. The 24% protein OVA fed group was not significantly suppressed at either day ($P > 0.05$ compared to SAL fed group). The 4% protein OVA fed group also was not suppressed at day 14, but showed significant suppression at day 21 ($P < 0.01$ compared to SAL fed group).

A similar pattern was observed in the 10 week group (Fig. 8.7), with only the 4% protein OVA fed group having suppressed IgM titres compared to the control group ($P < 0.05$) at day 14. No suppression was observed at day 21. The response of the 24% protein OVA fed group again was not suppressed at either day ($P > 0.05$ compared to SAL fed group).

II. IgG

The results obtained in this section were similar to those when the old method of ELISA was used. The slight differences which exist probably arose because of the increased efficiency of detection of IgG antibodies in the new method.

The results in Figure 8.8 show that 4% protein SAL fed OVA immunized mice had significantly less antibody at both d.14 and d.21 compared to the equivalent 24% protein group ($P < 0.05$). Feeding OVA before immunization significantly reduced antibody titres in both 4% and 24% protein groups at day 21 only ($P < 0.05$). At both d.14 and 21, the responses of 4% protein OVA fed mice were significantly less than those of 24% protein OVA fed mice ($P < 0.01$).

Figure 8.9 shows the antibody responses of mice maintained on the diets for 10 weeks before feeding antigen. 4% protein SAL fed OVA immunized mice had normal antibody titres at d.14 and d.21 compared to 24% protein mice ($P > 0.05$). Feeding OVA did not suppress the response of 24% protein mice ($P > 0.05$ compared to SAL fed group), however the response of 4% protein mice was suppressed ($P < 0.01$ at d.14, $P = 0.05$ at d.21 compared to SAL fed group).

Comments

By using the new ELISA procedure, the effect of protein deprivation on the induction of oral tolerance for both IgM and IgG antibody has been examined.

The IgM responses of 24% protein mice were not suppressed by feeding, in contrast to the responses of 4% protein mice. After short term deprivation, the IgG responses of 4% protein mice showed very profound suppression compared to 24% protein mice. After longer periods, the response of 4% protein mice was suppressed by feeding, but this did not occur in 24% protein mice.

The results suggest that the more profound tolerance for antibody responses in protein deprived animals extends to both IgM and IgG isotypes.

C. THE EFFECT OF CONTINUOUS PROTEIN DEPRIVATION ON ORAL TOLERANCE TO OVA IN BALB/c MICE

In a previous chapter, it was shown that mice from the inbred BALB/c strain could adapt better, in terms of their

weight and physical appearance, to a reduced protein intake, than could the hybrid strain (BDF_1) used for the majority of the experiments. The aim of this section was to examine if the modulating effect of protein deprivation on the pattern of oral tolerance was universal, or if it was just a characteristic of this hybrid strain under study.

Experimental protocol

Groups of female BALB/c mice were maintained on the diets for 2 weeks from weaning, before receiving a feed of antigen (d.-7: Fig. 8.1). The groups were maintained on the same diet for the remainder of the experiment.

Antibody responses

Figure 8.10 shows the IgM and IgG anti-OVA antibody responses at d.14 and d.21 after immunization. Very low levels of IgM were produced at d.14 and d.21 in all groups, with no significant difference existing between any group at any time.

4% protein OVA fed mice which were immunized with SAL/CFA showed small amounts of IgG antibody at both d.14 or d.21. There was no difference in IgG responses between the 4% and 24% protein SAL fed OVA immunized groups at either d.14 or d.21 ($P > 0.05$). Feeding OVA to 4% protein mice significantly suppressed the response at d.14 ($p = 0.01$) but not at d.21 ($P > 0.05$), while feeding OVA to 24% protein mice significantly suppressed the response at both d.14 and d.21 ($p = 0.01$).

In addition, the IgG responses of the tolerant groups of 4% and 24% protein mice were similar ($P > 0.05$) at both d.14 and d.21.

DTH responses

The results presented in Figure 8.11 indicate that the DTH response to OVA was depressed by protein deprivation. Therefore, the extent of the suppression induced by feeding could not be assessed in these mice. Feeding OVA to 24% protein mice prior to immunization resulted in 89% suppression ($P < 0.01$ compared to SAL fed group) of the subsequent DTH response.

Comments

The effect of protein deprivation on orally induced tolerance for antibody responses was different according to which strain of mouse was used. The results from this section indicate that, in contrast to BDF_1 mice, protein deprived BALB/c mice did not show more profound suppression of antibody responses after feeding. The normal expression of tolerance in these mice may be related to the increased ability of BALB/c mice to adapt to protein restriction in terms of weight and physical appearance.

D. ORAL TOLERANCE TO OVA IN NUTRITIONALLY REHABILITATED BDF_1 MICE

So far, it has proved impossible to examine the effect of short term protein deprivation on oral tolerance for DTH responses, as control 4% protein OVA immunized mice could not mount a positive response upon challenge with the antigen. However, the adverse effect which protein deprivation has on this response can be rapidly reversed by feeding with a diet containing normal levels of protein (Sakamoto *et al.*, 1979).

The aim of this section, therefore, was to examine the effect of short term protein deprivation on DTH oral tolerance by nutritionally rehabilitating protein deprived mice immediately after feeding antigen and one week before immunization. It was hoped that this would restore the potential for DTH responsiveness in these mice and that only the induction of tolerance would be affected by protein deprivation.

Experimental protocol

Female BDF_1 mice were weaned onto the 4% protein or 24% protein diets at 3 weeks of age and were maintained on these diets for 2 weeks. Groups of animals were fed either 25 mg OVA or 0.2 ml SAL (d.-7: Fig. 8.1), after which time the 4% protein mice were switched onto the 24% protein diet. Animals on the normal diet already, remained on this diet for the duration of the experiment. One week later, all groups were immunized with 100 µg OVA/CFA and the experiment followed the standard protocol as before (Fig. 8.1).

Antibody responses

The primary antibody responses of normal and nutritionally rehabilitated mice fed either OVA or SAL before immunization are shown in Figure 8.12.

I. IgM

4% → 24% protein SAL fed mice showed normal responses to OVA at both d.14 and d.21 compared to 24% protein SAL fed mice ($P > 0.05$). OVA fed mice from both dietary groups had suppressed responses at d.21 ($P < 0.01$ compared to control SAL fed groups). There were no differences in responses between the OVA fed groups at d.14 or d.21 ($P > 0.05$).

II. IgG

In a similar fashion, 4% → 24% protein SAL fed mice had normal IgG responses at d.14 and d.21 compared to 24% protein SAL fed mice ($P > 0.05$). Feeding OVA before immunization in both dietary groups significantly suppressed responses at d.14 and d.21 ($P < 0.01$ compared to control SAL fed groups). There were no differences in responses between the OVA fed groups at d.14 and d.21 ($P > 0.05$).

DTH responses

Figure 8.13 shows the DTH responses of normal and nutritionally rehabilitated mice fed either OVA or SAL prior to immunization. There was no difference between the response of 24% protein SAL fed mice and that of 4% → 24% protein SAL

fed mice upon challenge with antigen ($P > 0.05$). The 24% protein OVA fed mice showed 95% suppression ($P < 0.01$) of their DTH response when compared to the SAL fed control group, however, the 4% → 24% protein OVA fed group only showed 44% suppression ($P < 0.05$) of their response compared to the nutritionally rehabilitated SAL fed control group. The difference in responses between the 4% → 24% protein and 24% protein OVA fed groups was also significant ($P < 0.01$).

Comments

The results indicate that starting nutritional therapy with a diet containing normal levels of protein immediately after feeding and one week before immunization restored the IgM and IgG antibody responses to normal. In addition, the level of tolerance for antibody responses induced by feeding antigen was of similar magnitude in both 4% → 24% and 24% protein groups. Thus, it would appear that for full expression of the more profound tolerance for antibody responses observed previously, it is necessary to continue the period of protein restriction past the time of antigen feeding.

The DTH response to OVA was also restored after nutritional therapy, thereby making it possible to examine the effect of short-term protein deprivation on the induction of DTH oral tolerance. The degree of tolerance in the nutritionally rehabilitated OVA fed group was decreased compared to that observed in the normal OVA fed group. It is interesting to

note however, that the abrogation was not complete as these animals still showed significant suppression of their DTH response.

E. THE EFFECT OF SHORT TERM PROTEIN RESTRICTION INITIATED AT MATURITY ON ORAL TOLERANCE TO OVA IN BDF_1 MICE

The time of weaning in mice has been identified as one of major upset in terms of oral tolerance induction for both antibody and DTH responses (see Chapter 2). When antigen is fed at or within a few days of weaning, tolerance for both antibody and DTH responses is impaired. This effect is dependent upon the proximity of the time of feeding to weaning.

Short term protein restriction, initiated at weaning, can restrict oral tolerance for DTH responses. In this section, a small pilot study was set up, in which animals were subjected to short term protein restriction only after they had reached maturity upon a normal diet. DTH oral tolerance was examined in these mice, and compared to the results obtained in the previous section.

Experimental protocol

Groups of female BDF_1 mice were weaned onto the 24% protein diet at 3 weeks of age, and were maintained on this diet for 4 weeks. They were then placed on the 4% protein diet for 2 weeks, after which time they received a feed of 25 mg OVA, 25 mg HSA and 0.2 ml SAL (d.-7: Fig. 8.1). Immediately after feeding, all groups were switched back

onto the 24% protein diet, and were continued on this diet for the remainder of the experiment. Animals were immunized with either 100 µg OVA/CFA or 0.05 ml SAL/CFA one week after feeding.

DTH responses

Figure 8.14 shows the DTH responses in mice which had been protein restricted for 2 weeks after they had reached maturity. In this case, short term protein deprivation had no effect on the level of DTH oral tolerance. The DTH response of these nutritionally rehabilitated mice was completely suppressed by feeding (100% suppression: $P < 0.01$).

Comments

Unfortunately, this study was not complete, as insufficient numbers of mice were available for 24% protein maintained SAL fed and OVA fed groups. However, it can be seen that short term protein deprivation did not restrict orally induced suppression of DTH, when itwas initiated at maturity. In contrast to this, the same period of deprivation can partially prevent the induction of tolerance when begun directly after weaning (Section D). The modulating effect of protein deprivation on oral tolerance may be determined not so much by the duration of restriction, but by the proximity of its initiation to weaning.

F. A COMPARISON OF THE EFFECT OF CYCLOPHOSPHAMIDE PRETREATMENT WITH SHORT TERM PROTEIN DEPRIVATION ON ORAL TOLERANCE FOR ANTIBODY AND DTH RESPONSES

It has previously been shown that treatment of mice with CY 2 days before feeding antigen can partially abrogate oral tolerance for DTH responses whilst having little effect on the tolerance observed for antibody responses (see Chapter 2). The experiment in Section D has demonstrated a similar effect of short term protein deprivation initiated at weaning on DTH oral tolerance. Accordingly, it was the purpose of this section to investigate whether CY or protein deprivation affected the same or different populations of cells by examining the relative and additive abilities of the two regimes to modulate the induction of oral tolerance.

Experimental protocol

Groups of mice were weaned onto the diets at 3 weeks of age, and were maintained on the diets for 2 weeks before receiving a feed of either 25 mg OVA or 0.2 ml SAL (d.-7: Fig. 8.1). Animals which had been maintained on the 4% protein diet were nutritionally rehabilitated immediately after feeding (Groups A, B and E). Groups B,D and E received 100 mg/kg CY i.p. 2 days before feeding, while the remaining groups were sham-injected with 0.1 ml SAL. All groups were immunized with 100 µg OVA/CFA one week after feeding.

Antibody responses

The responses of the SAL fed group E were taken as the control values for the experiment, as it has previously been shown that nutritional rehabilitation after feeding and before immunization restores the IgM and IgG responses to normal. In addition, pretreatment of a control SAL fed group with CY does not affect subsequent systemic immune responses (Strobel, 1983).

I. IgM

Only trace amounts of IgM were apparent at day 14, and there was no difference in levels between any of the five experimental groups ($P > 0.05$: Fig. 8.15). At day 21, feeding OVA before immunization did not affect the responses of Groups C and D ($P > 0.05$), but suppressed the responses of group A and B ($P < 0.01$ and $P < 0.05$ respectively compared to group E).

II. IgG

At day 14 post immunization, the response of group B was the only one which was suppressed by feeding relative to group E ($P < 0.05$: Fig. 8.16). However, at day 21, the responses of both group A and group B were suppressed ($P < 0.01$ and $P < 0.05$ respectively). Feeding OVA before immunization did not affect the responses of groups C and D at either day examined ($P > 0.05$).

DTH responses

The response of group E was again taken as the positive control value (Fig. 8.17). Group C was taken as the control tolerant group and showed 98% suppression ($P < 0.01$) of the DTH response to OVA compared to group E. The responses of groups A and D were not significantly different ($P > 0.05$) from that of group E. However, the response of group B was significantly suppressed (40% suppression: $P < 0.01$). On comparing the effect of the three treatment regimes used in groups A, B and D, there was no difference in responses between any of these groups ($P > 0.05$). Finally, the response of the control tolerant group C was significantly less ($P < 0.01$) than those of the three experimental groups A, B and D.

Comments

The results indicate that the only groups in the experiment which showed tolerance for IgM and IgG antibody responses induced by feeding were the ones which had been protein deprived for 2 weeks before feeding, regardless of whether they were pretreated with CY or not. CY itself, given 2 days before oral administration of antigen had no effect on the induction of tolerance for antibody responses. In this experiment, feeding antigen to 24% protein maintained mice (group C) did not result in significant suppression of antibody responses compared to group E, however, this may have been a result of the low number of serum samples taken from this group.

When comparing the effect of protein deprivation with CY on oral tolerance for DTH responses, a clearer picture is observed. The three treatment regimes used in this experiment (protein deprivation alone, CY alone, and combined protein deprivation and CY) all resulted in partial abrogation of DTH oral tolerance, with there being no difference in the responses of the three groups upon challenge with antigen.

It is reasonable to assume that short term protein deprivation and CY can adversely affect the same population of T_s cells which are responsible for the orally-induced suppression of DTH responses. Furthermore, protein deprivation has an additional effect in terms of tolerance for antibody responses.

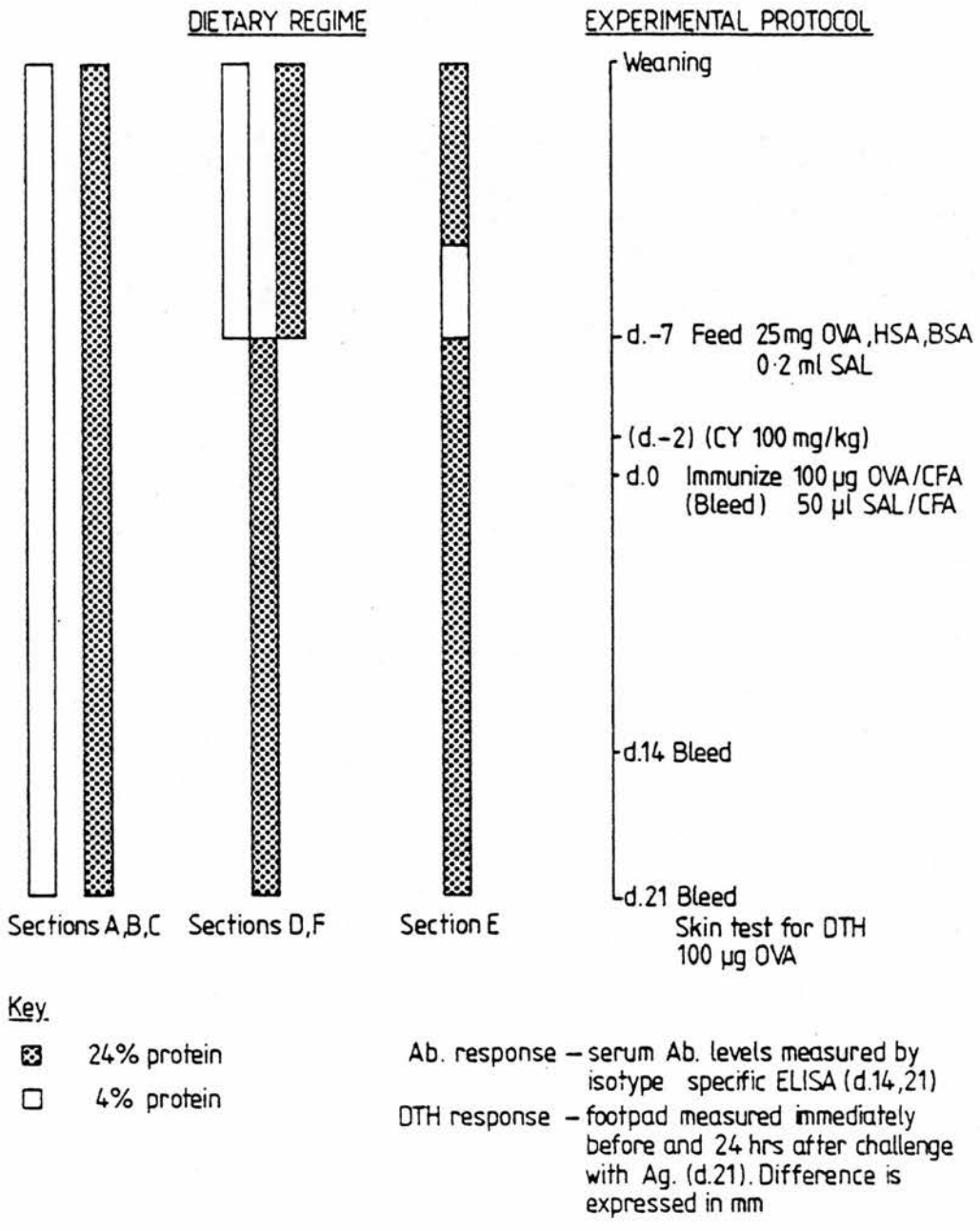

Fig. 8.1: General experimental protocol to examine the induction of tolerance for antibody and DTH responses by a single feed of antigen.

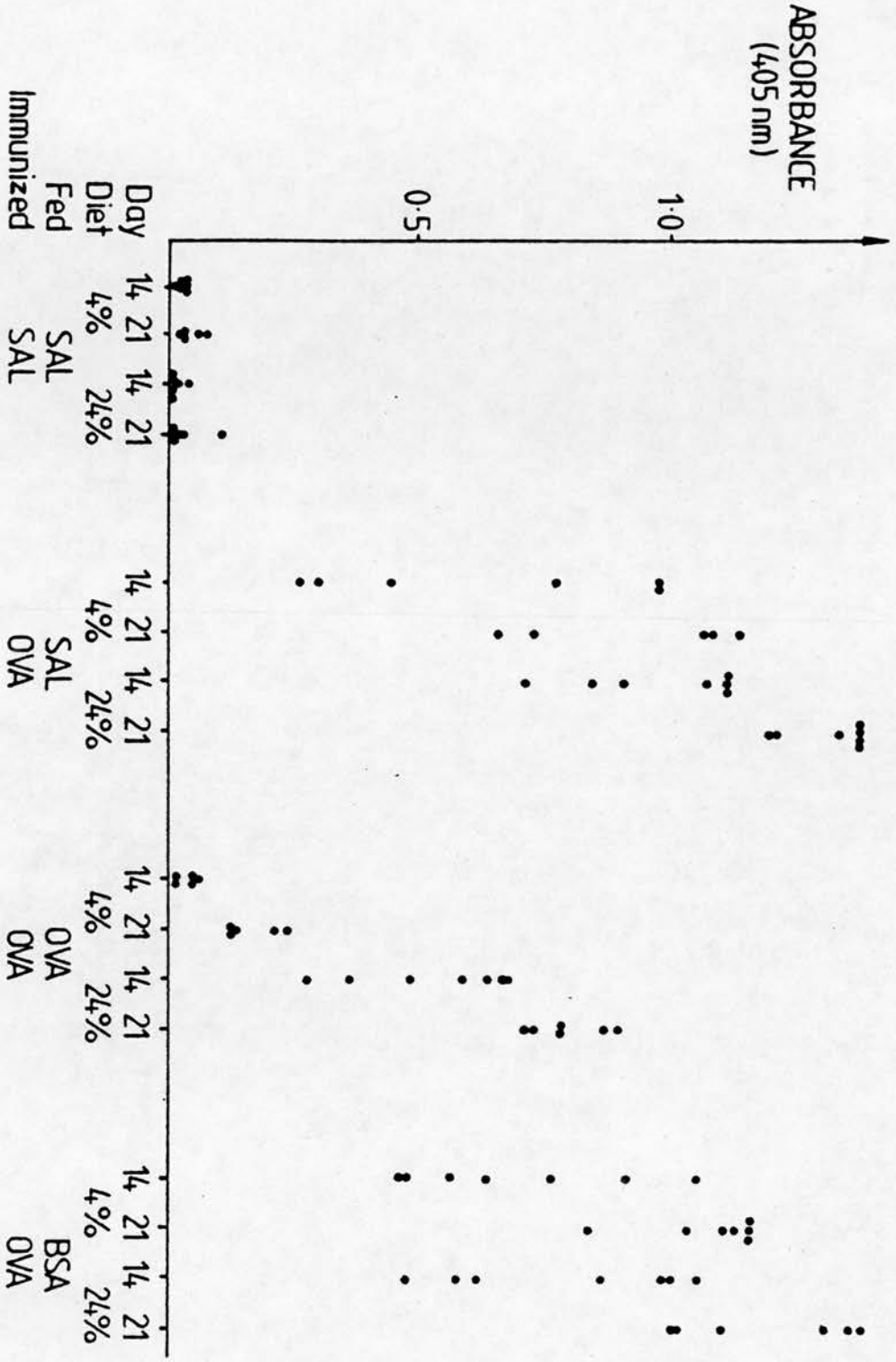

Fig. 8.2: Primary IgG antibody responses of BDF_1 mice maintained on the 4% or 24% protein diets for 2 weeks prior to feeding antigen. Animals were immunized 1 week after feeding and were continued on the same diet as before. Antibody levels measured by the old method of ELISA. Statistical comparisons are contained in the text.

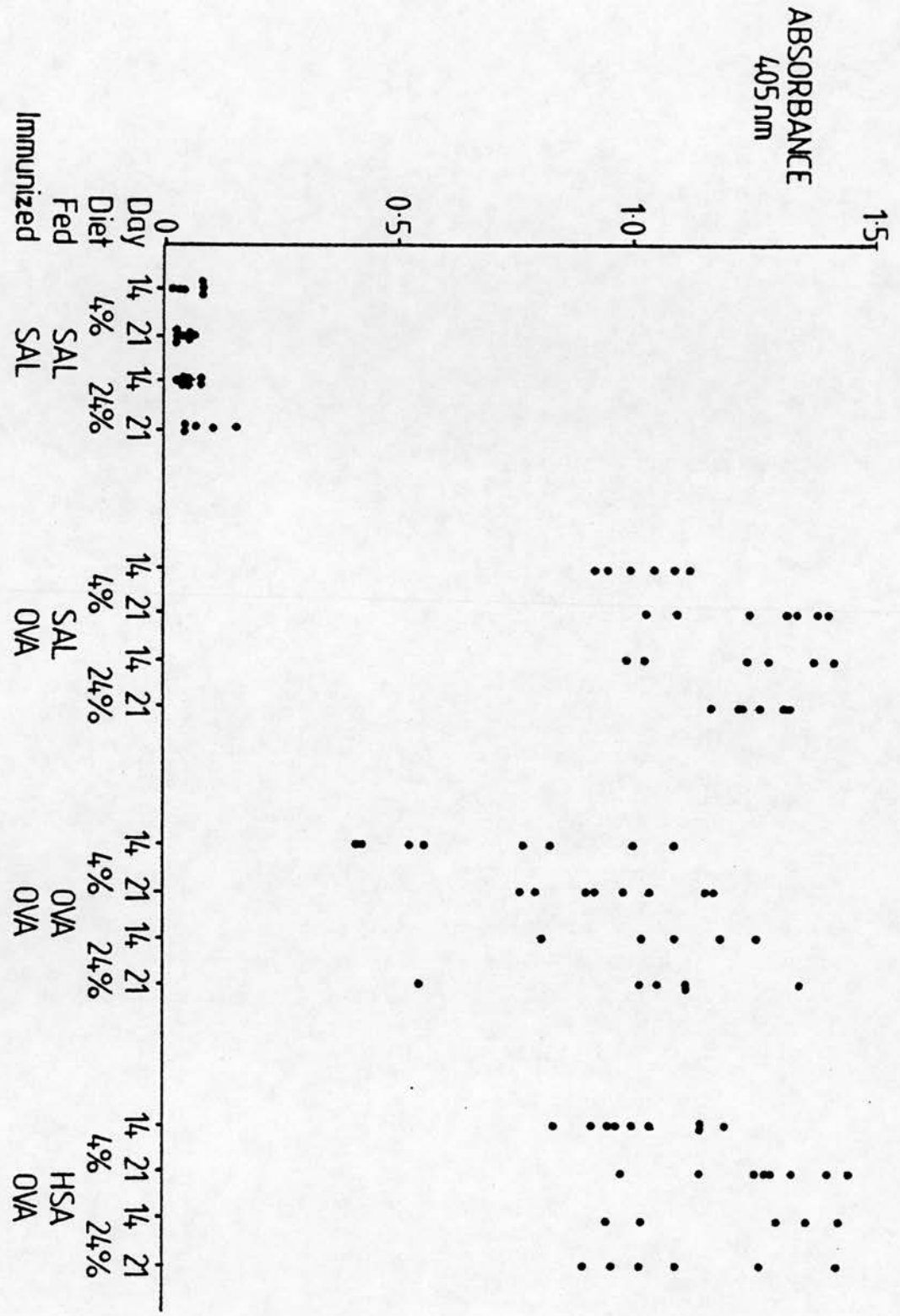

Fig. 8.3: Primary IgG antibody responses of BDF_1 mice maintained on the 4% or 24% protein diets for 10 weeks prior to feeding antigen. Animals were immunized 1 week after feeding, and were continued on the same diet as before. Antibody levels measured by the old method of ELISA. Statistical comparisons are contained in the text.

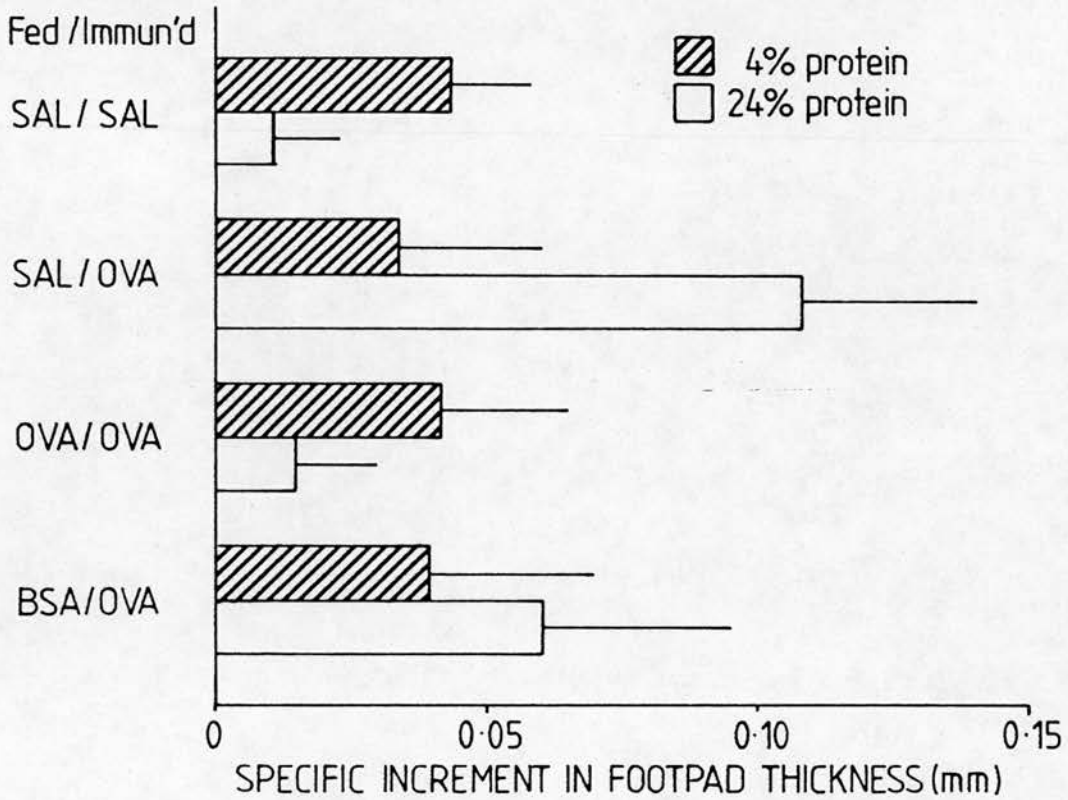

Fig. 8.4: DTH responses of BDF_1 mice maintained on the 4% or 24% protein diets for 2 weeks prior to feeding antigen. Animals were immunized 1 week after feeding, and were continued on the same diet as before. DTH was assessed 21 d. after immunization and the bars represent the mean + 1 s.d. of 4–8 mice. Statistical comparisons are contained in the text.

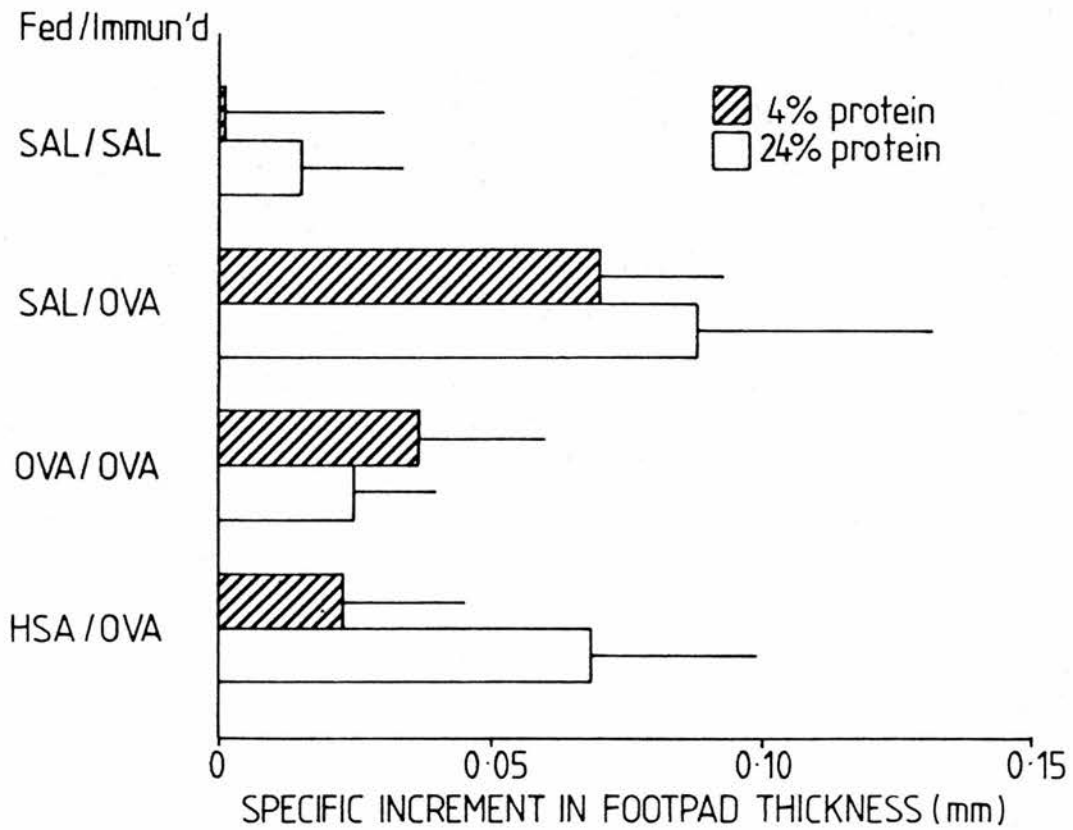

Fig. 8.5: DTH responses of BDF_1 mice maintained on the 4% or 24% protein diets for 10 weeks prior to feeding antigen. Animals were immunized 1 week after feeding, and were continued on the same diet as before. DTH was assessed 21 d. after immunization, and the bars represent the mean + 1 s.d. of 4-8 mice. Statistical comparisons are contained in the text.

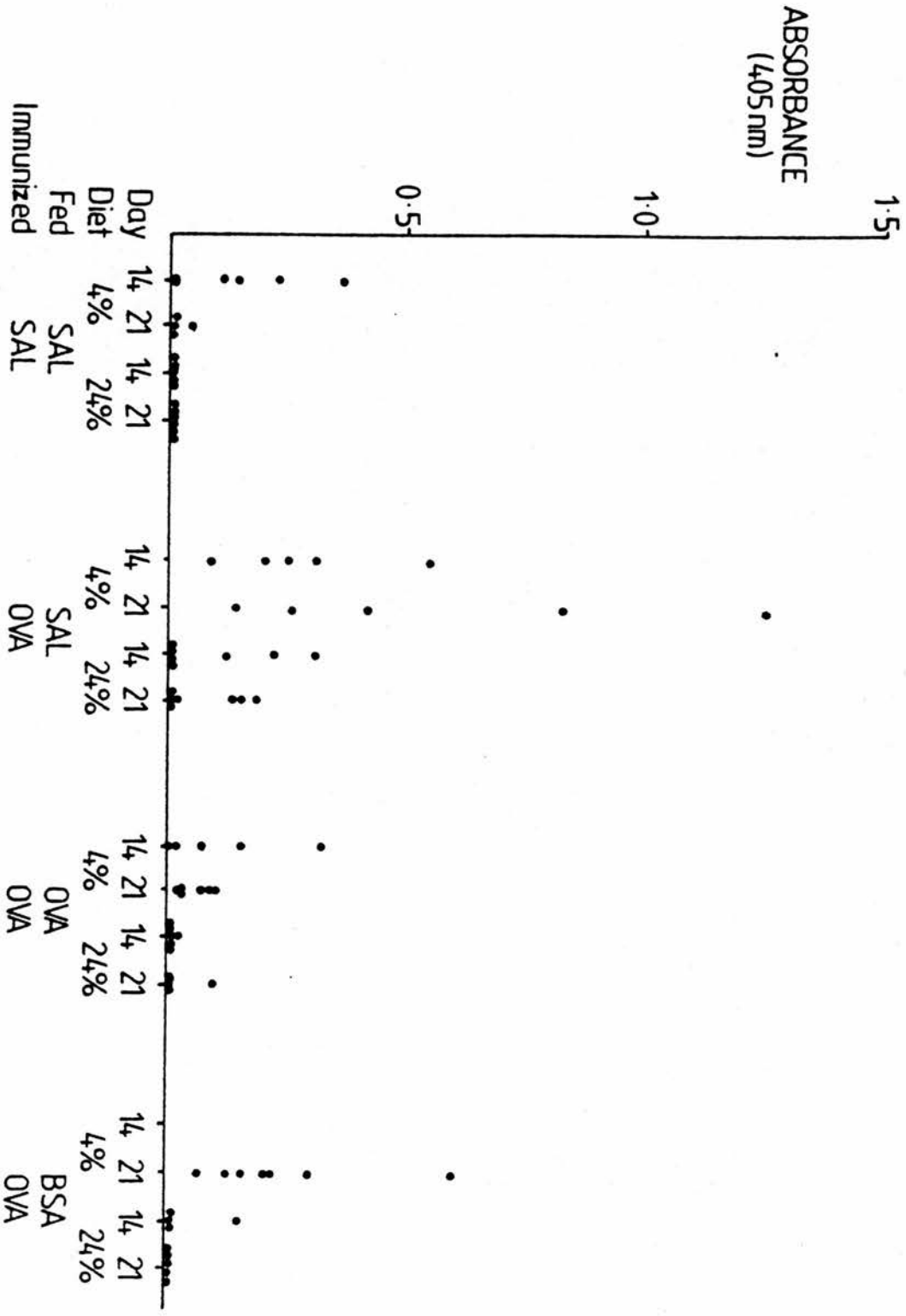

Fig. 8.6: Primary IgM antibody responses of BDF_1 mice maintained on the 4% or 24% protein diets for 2 weeks prior to feeding antigen. Animals were immunized 1 week after feeding and were continued on the same diet as before. Antibody levels measured by the new method of ELISA. Statistical comparisons are contained in the text.

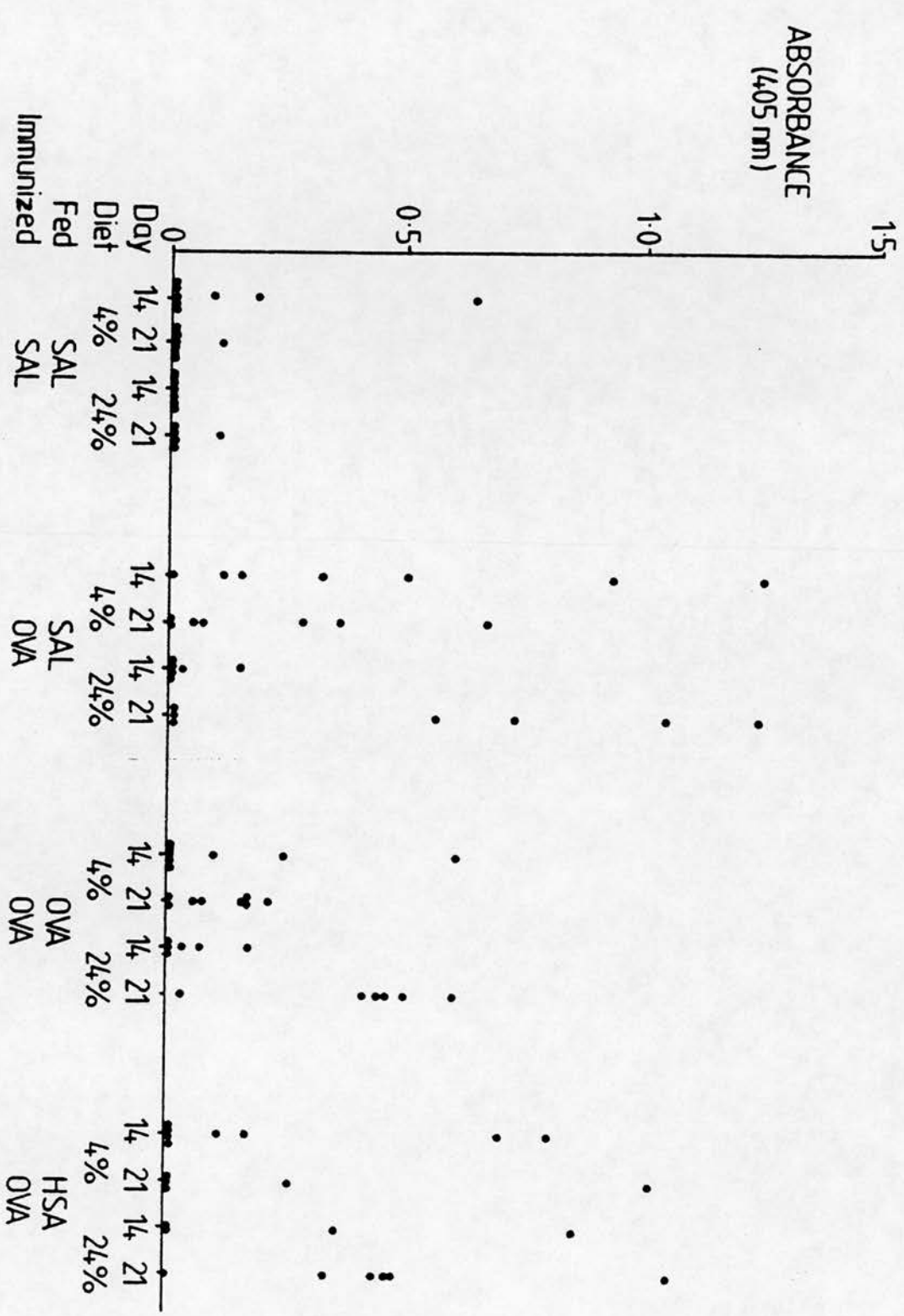

Fig. 8.7: Primary IgM antibody responses of BDF_1 mice maintained on the 4% or 24% protein diets for 10 weeks prior to feeding antigen. Animals were immunized 1 week after feeding and were continued on the same diet as before. Antibody levels measured by the new method of ELISA. Statistical comparisons are contained in the text.

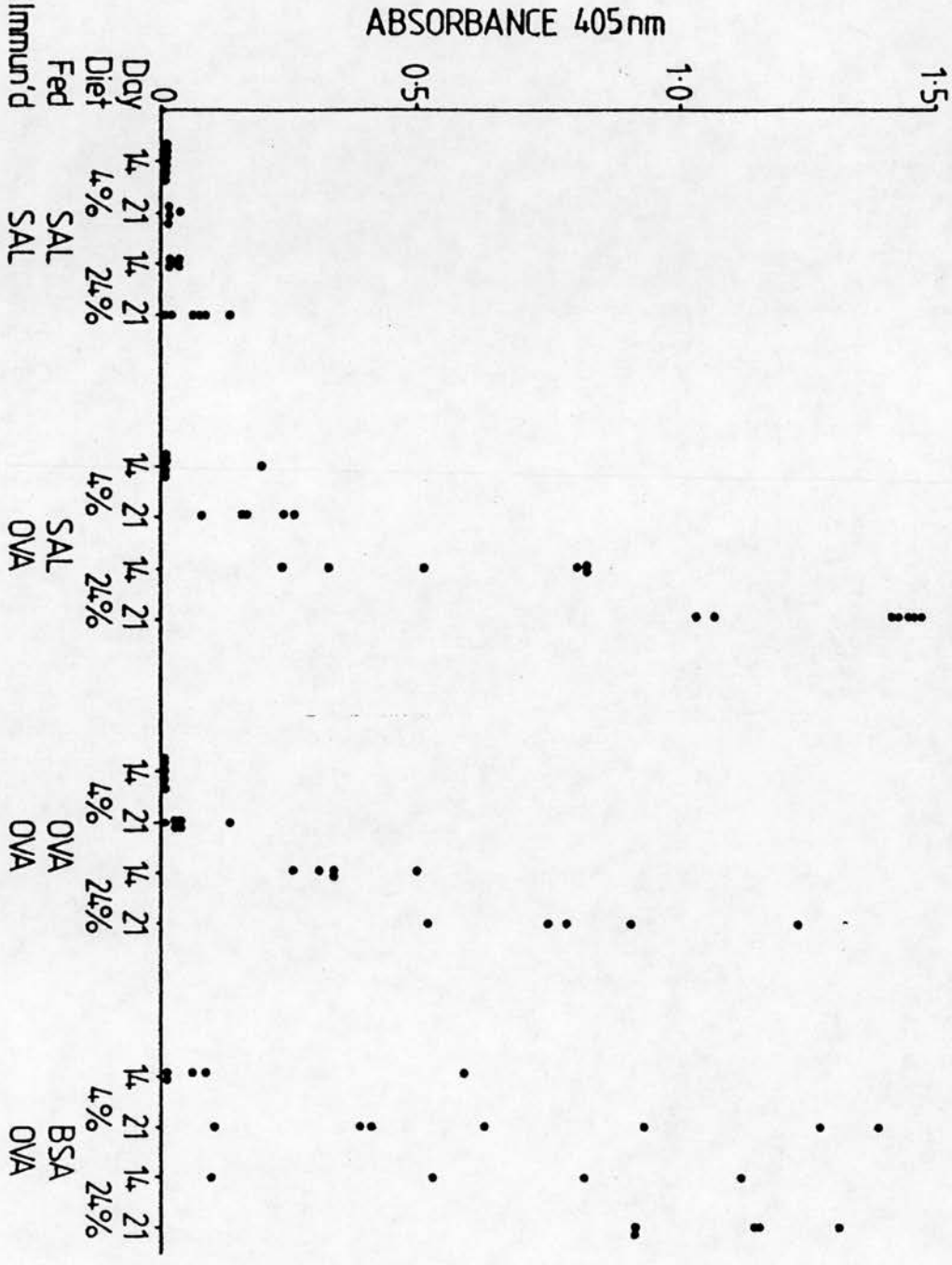

Fig. 8.8: Primary IgG antibody responses of BDF_1 mice maintained on the 4% or 24% protein diets for 2 weeks prior to feeding antigen. Animals were immunized 1 week after feeding and were continued on the same diet as before. Antibody levels measured by the new method of ELISA. Statistical comparisons are contained in the text.

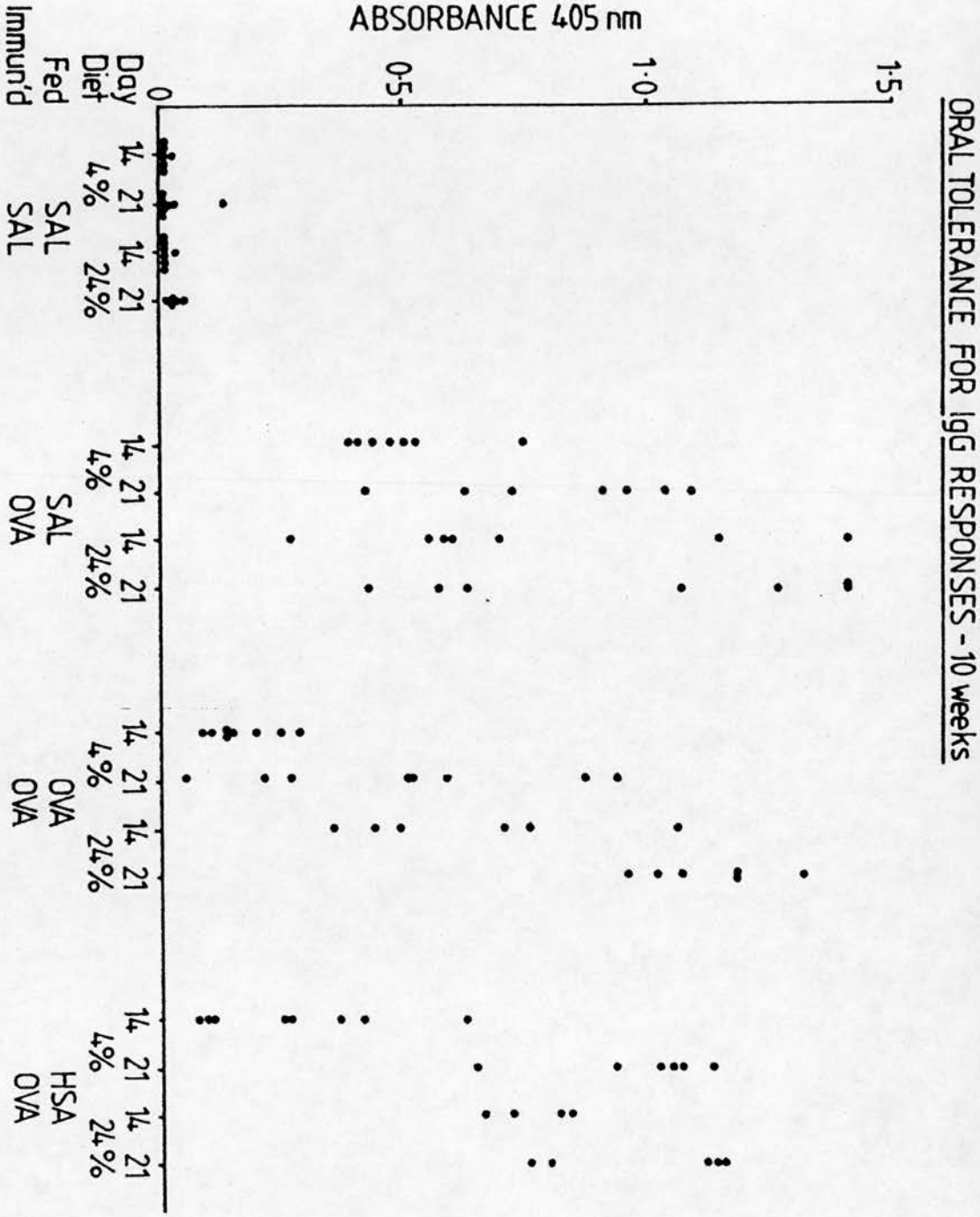

Fig. 8.9: Primary IgG antibody responses of BDF_1 mice maintained on the 4% or 24% protein diets for 10 weeks prior to feeding antigen. Animals were immunized 1 week after feeding and were continued on the same diet as before. Antibody levels measured by the new method of ELISA. Statistical comparisons are contained in the text.

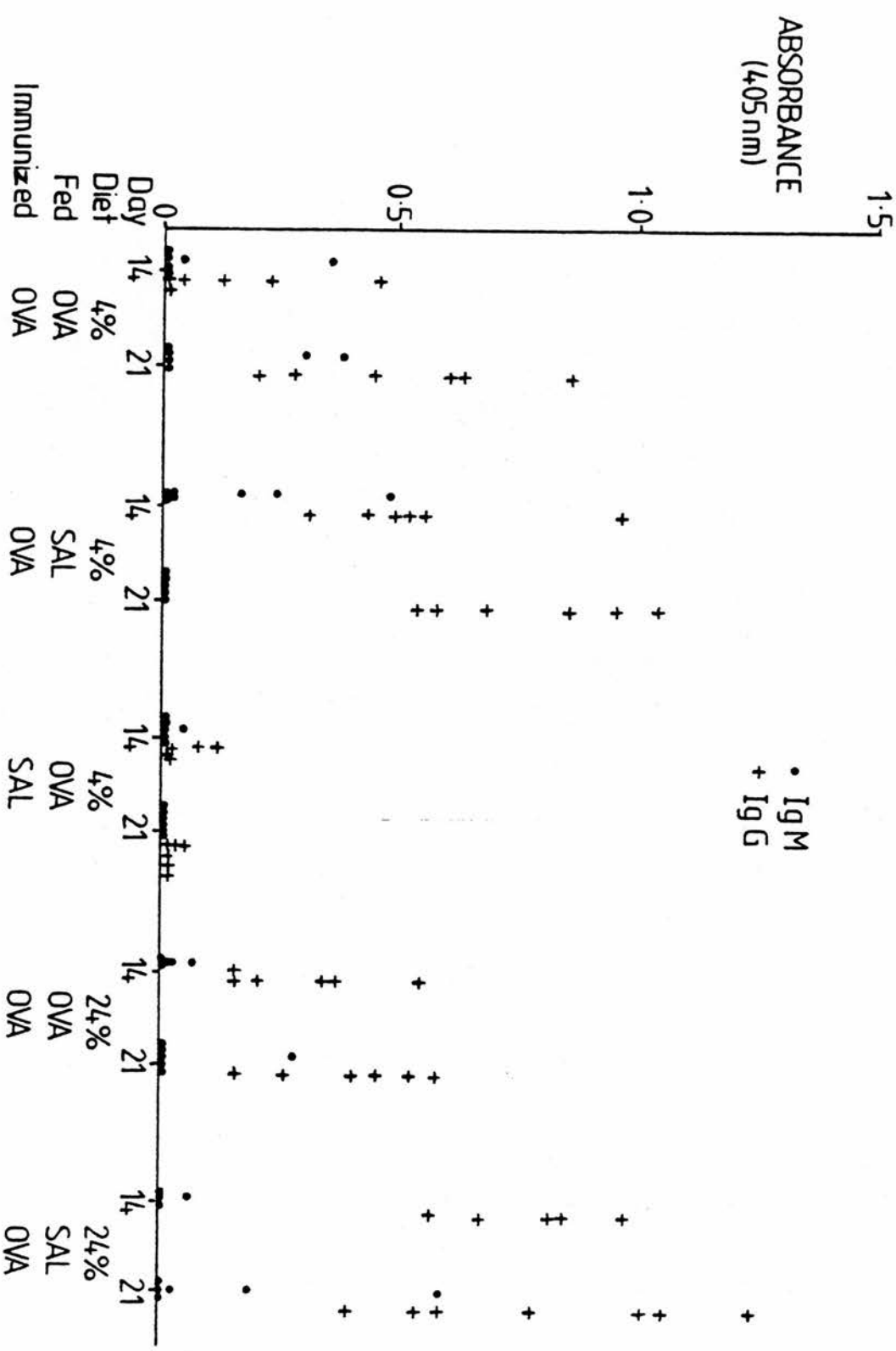

Fig. 8.10: Primary IgM and IgG antibody responses of BALB/c mice maintained on the 4% or 24% protein diets for 2 weeks before feeding antigen. Animals were immunized 1 week after feeding and were continued on the same diet as before. Statistical comparisons are contained in the text.

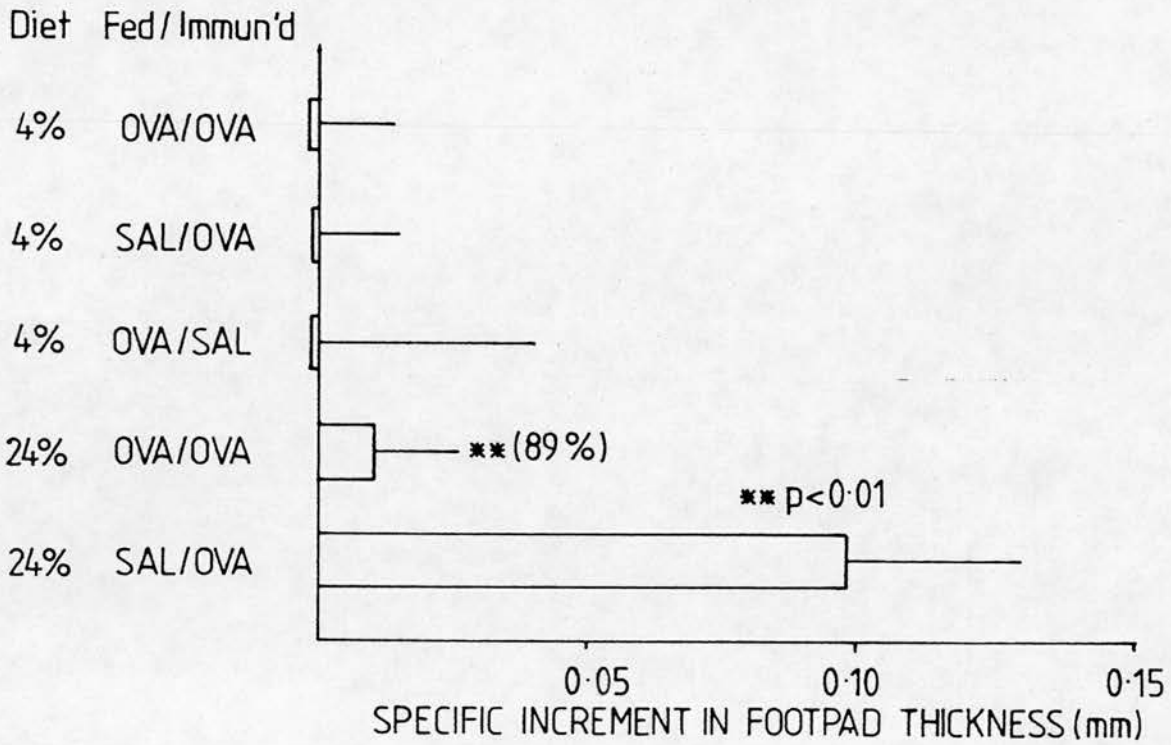

Fig. 8.11: DTH responses of BALB/c mice maintained on the 4% and 24% protein diets for 2 weeks prior to feeding antigen. Animals were immunized 1 week after feeding and were continued on the same diet as before. DTH was assessed 21 d. after immunization, and the bars represent the mean + 1 s.d. of 6–8 mice. Figure in bracket represents % suppression of the response of 24% OVA fed group vs. SAL fed control group.

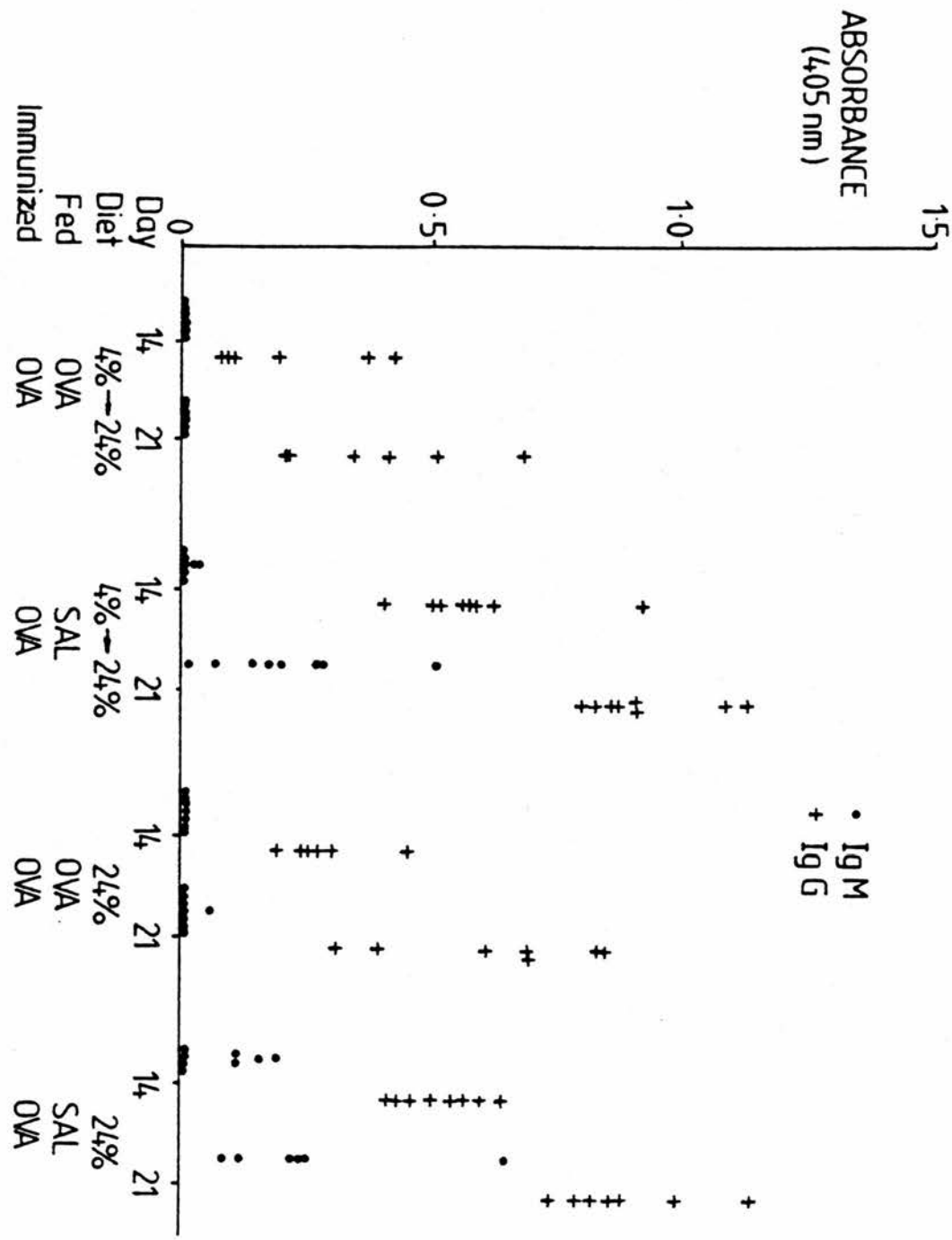

Fig. 8.12: Primary IgM and IgG antibody responses of BDF_1 mice which had received nutritional therapy immediately after feeding antigen. Control animals received the 24% protein diet throughout. All groups were immunized 1 week after feeding. Statistical comparisons are contained in the text.

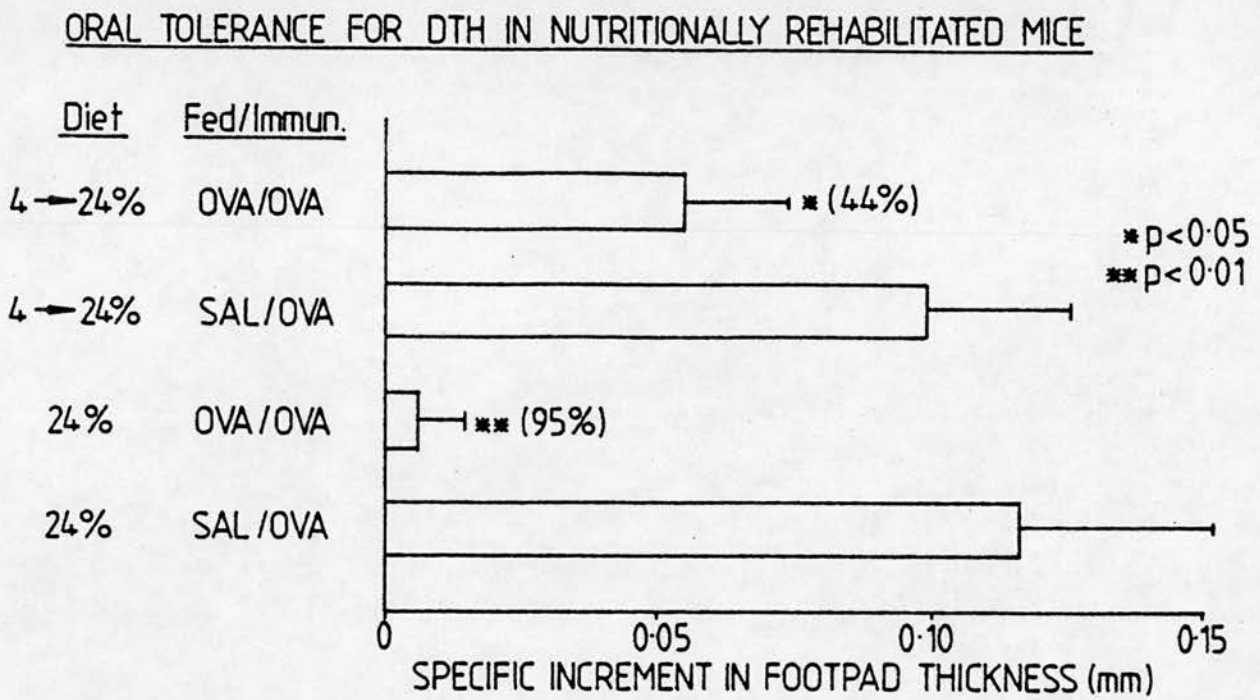

Fig. 8.13: DTH responses of BDF_1 mice which had received nutritional therapy immediately after feeding. Control animals received the 24% protein diet throughout. All groups were immunized 1 week after feeding and DTH was assessed 21 d. after immunization. Bars represent mean + 1 s.d. of 6-8 mice. Figures in brackets are % suppression of OVA fed group vs. SAL fed dietary control group.

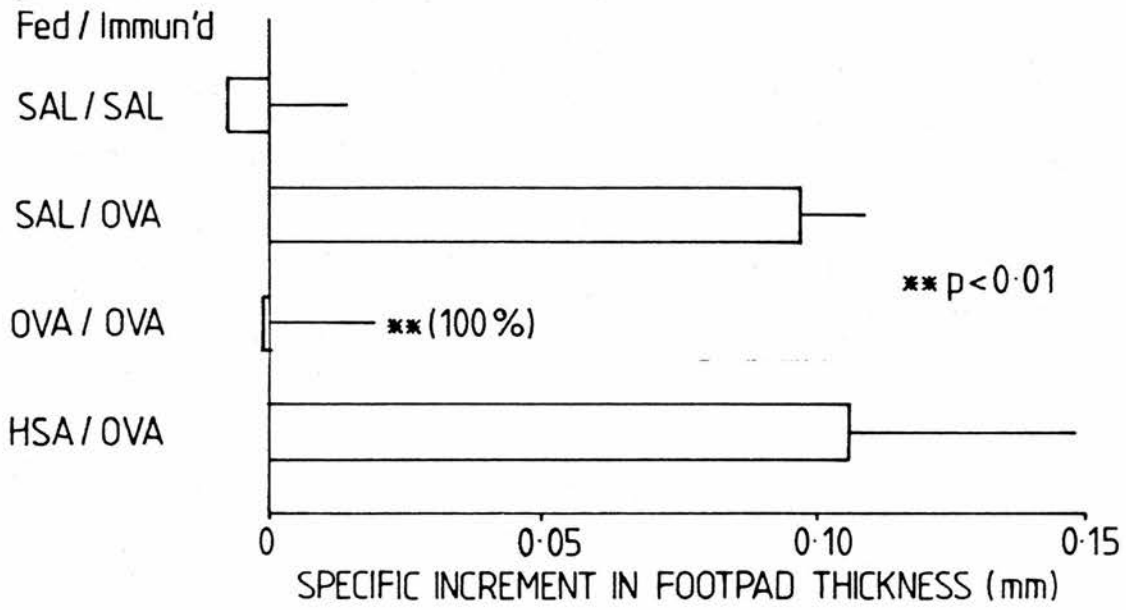

Fig. 8.14: DTH responses of BDF_1 mice where 2 weeks protein restriction was initiated at maturity. Nutritional therapy was started immediately after feeding antigen, and 1 week before immunization. DTH was assessed 21 d. after immunization and the bars represent the mean + 1 s.d. of 6-8 mice. Figure for % suppression (OVA fed vs. SAL fed) given in brackets.

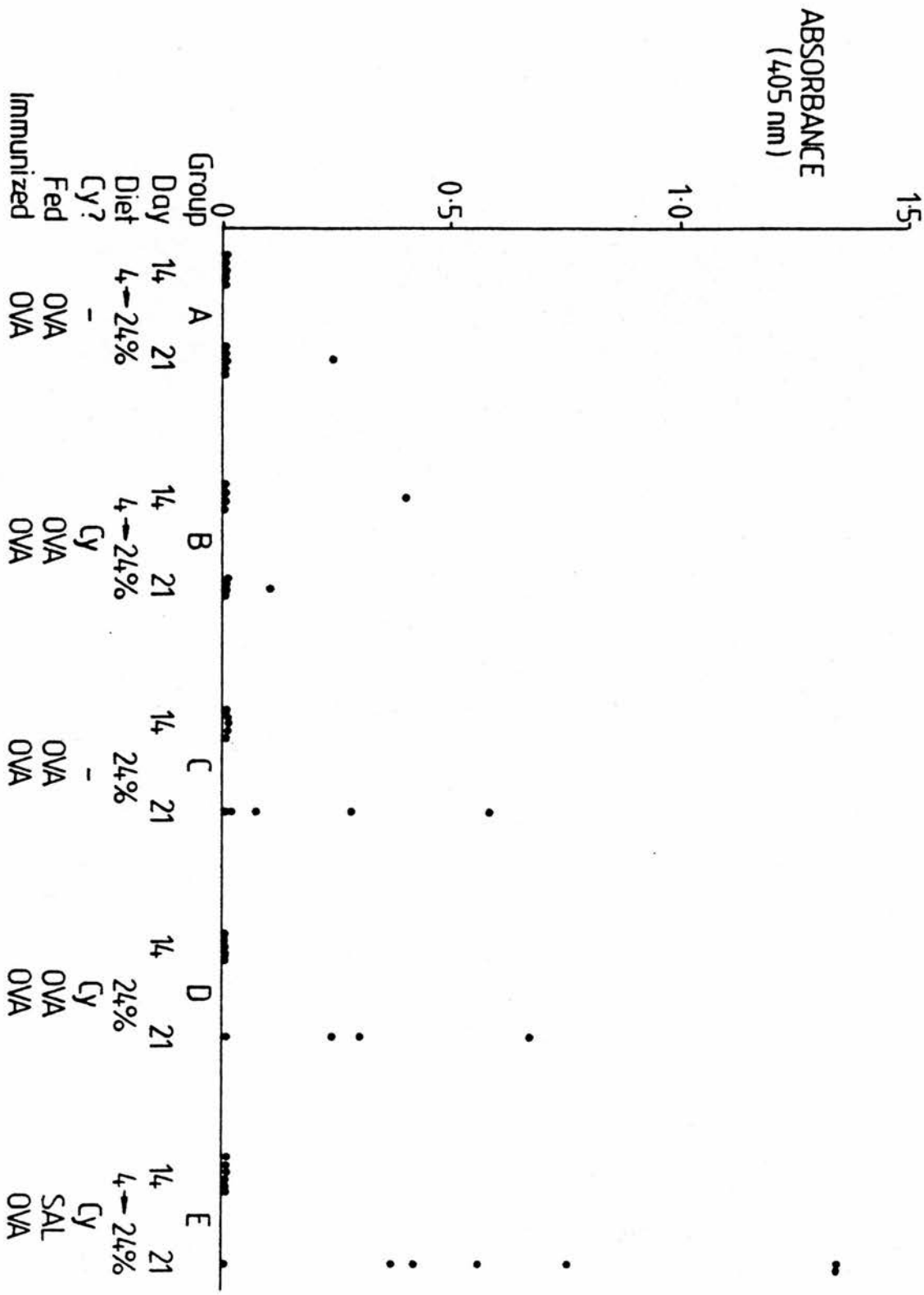

Fig. 8.15: Primary IgM antibody responses of BDF_1 mice protein restricted for 2 weeks before feeding antigen (grps. A, B and E). Nutritional therapy was begun immediately after feeding and groups were immunized 1 week later. Groups C and D were maintained on the 24% protein diet throughout. Groups B, D and E were 100 mg/kg CY pretreated 2 d. before feeding. Statistical comparisons are contained in the text.

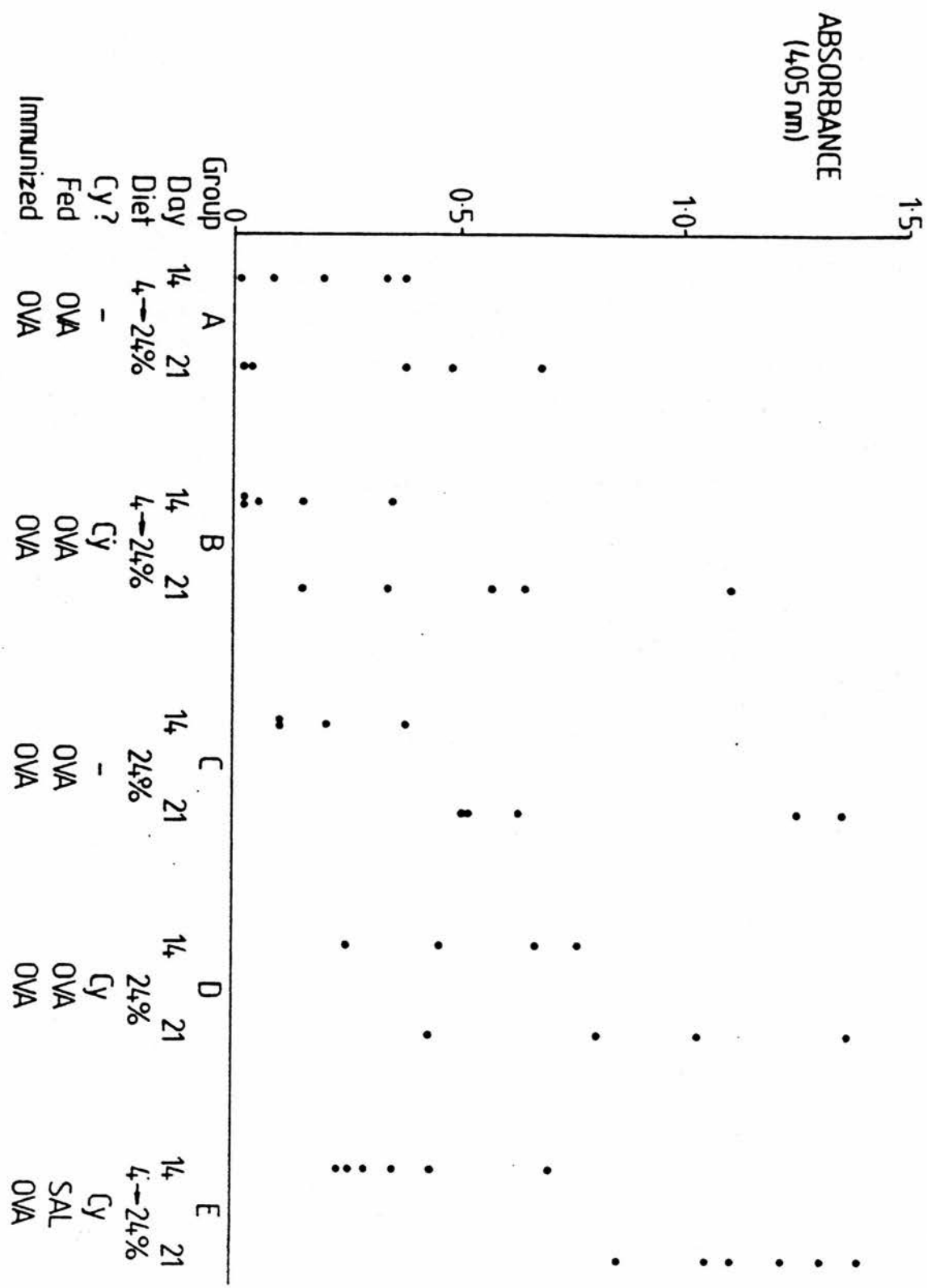

Fig. 8.16: Primary IgG antibody responses of BDF_1 mice protein restricted for 2 weeks before feeding antigen (grps. A, B and E). Nutritional therapy was begun immediately after feeding, and groups were immunized 1 week later. Groups C and D were maintained on the 24% protein diet throughout. Groups B, D and E were 100 mg/kg CY pretreated 2 d. before feeding. Statistical comparisons are contained in the text.

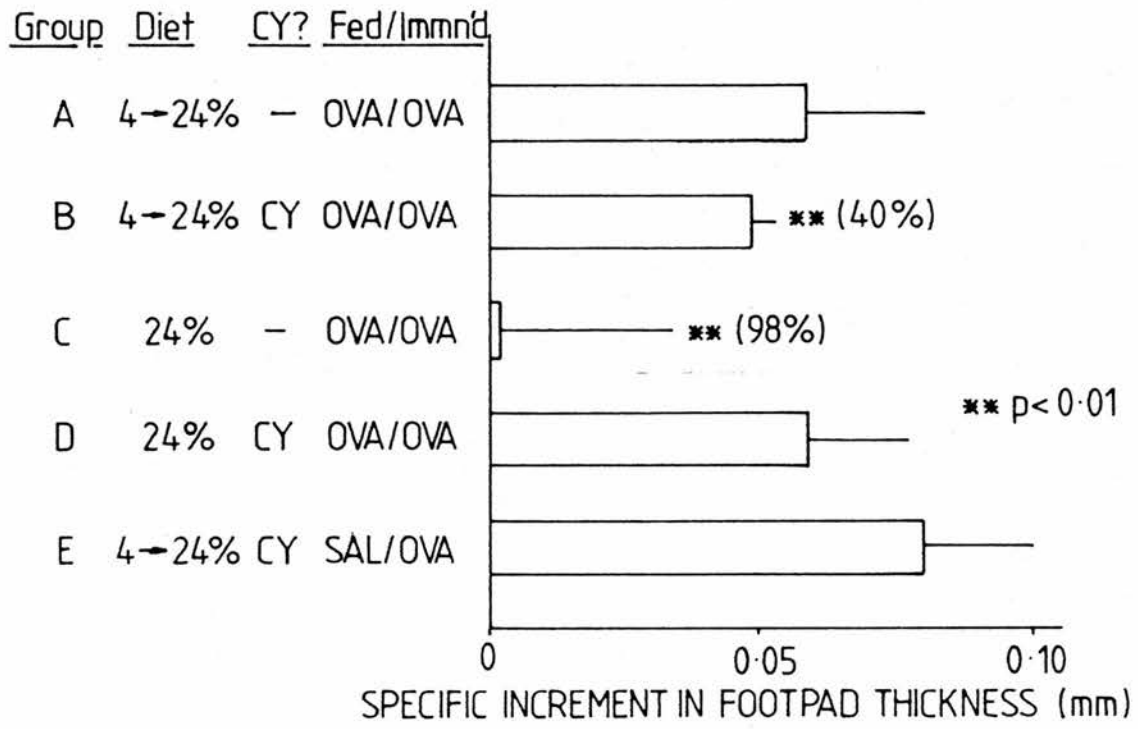

Fig. 8.17: DTH responses of BDF_1 mice protein restricted for 2 weeks (grps. A, B and E) before feeding antigen. Nutritional therapy was begun immediately after feeding and groups were immunized 1 week later. Groups C and D were maintained on the 24% protein diet throughout. Groups B, D and E were 100 mg/kg CY pretreated 2 d. before feeding. DTH was assessed 21 d. after immunization and bars represent the mean + 1 s.d. of 5-8 mice. Figures in brackets represent % suppression grps. B and C vs. control group E.

CHAPTER 9

TRANSFER OF TOLERANCE FROM PROTEIN DEPRIVED MICE

Introduction to experiments

The transfer of tolerance for systemic immune responses to naive animals has been accomplished by using both lymphoid cells and serum from orally immunized mice (see Chapter 2). These types of experiments are important in defining mechanisms responsible for suppression of antibody and DTH responses. Furthermore, adoptive transfer experiments can help to identify where the breakdown in suppressive mechanisms may occur, in conditions where tolerance is altered.

Several factors may be responsible for the abnormal pattern of tolerance observed in protein deprived animals. For example, the number and function of suppressor cells within the protein deprived host may be reduced. Alternatively, processing of antigen by the intestine may be altered such that tolerogenic moieties of the molecule are not produced. Finally, differences in quantity of antigen adsorbed across the gut may lead to immunological priming and not tolerance. The aim of this section, therefore, was to further examine the mechanisms responsible for the altered pattern of oral tolerance induction by using cell and serum transfer protocols. In addition, transport of antigen from the gut to the circulation of protein deprived and normal mice was assessed.

Transfer of tolerance with cells from OVA fed donors

The experiments in this section were in two parts. First, the efficiency of the cell transfer protocol in my hands was examined using mice which had been maintained on

the normal laboratory diet. Included in this trial were experimental and control cell recipient groups and, in addition, control groups which assessed the procedures used to induce tolerance. Second, the effect of protein deprivation on the ability of spleen cells from OVA fed donors to transfer tolerance to syngeneic recipients was examined.

A. CELL TRANSFER Part 1

Experimental protocol

The protocol used to compare the extent of orally induced tolerance in vivo with the ability of fed animals to transfer that tolerance is shown in Table 9.1. The effect of CY on oral tolerance and transferable suppression was also examined. Male BDF_1 mice, aged 6-8 weeks, were i.p. injected with 100 mg/kg CY or 0.1 ml SAL (d.-9), and two days later received a feed of 25 mg OVA or 0.2 ml SAL according to the protocol. One week after feeding, cell donor animals were sacrificed, their spleens removed, and 10^8 viable cells injected i.p. into syngeneic male recipients aged 6-8 weeks. Approximately 4 hours later, all groups (both recipient (grps. A-C) and control (groups D-G)) were immunized i.d. into one rear footpad with 100 µg OVA/CFA. Serum antibody levels were assessed 2 and 3 weeks after immunization. All groups were skin tested for DTH responses 21 days after immunization by challenging the contralateral footpad with 100 µg OVA/SAL. The footpad was measured immediately before, and 24 hours after challenge and the difference expressed in mm.

Antibody responses

I. IgM

Interpretation of the results of control groups D-G were complicated by the fact that group G had increased levels of IgM over the positive control group D at day 14 ($P < 0.01$) (Fig. 9.1). The reason for this anomaly was unknown. There was no difference between the IgM responses of groups D, E and F at either d.14 or d.21 ($P > 0.05$). At day 14, the responses of the three cell transfer groups were similar (groups A, B and C: $P > 0.05$). However, at day 21, group B (OVA fed cells) had decreased levels of antibody compared to group A (SAL fed cells) and group C (CY/OVA fed cells $P < 0.05$).

II. IgG

The results in Figure 9.2 indicate that Group E (OVA fed) mice had decreased IgG responses at both d.14 and d.21 compared to SAL fed group D ($P < 0.01$). Furthermore, this suppression was unaffected by CY pretreatment as group F had similarly decreased responses at d.14 and d.21 compared to group D ($P < 0.01$). There was no difference in IgG levels between any of the three cell transfer groups at d.21 ($P > 0.05$), however recipients of cells from OVA fed donors did show suppressed responses at d.14 (Group B vs. Group A: $P < 0.05$). This suppression was not evident in group C (CY/OVA fed cells). Group A (SAL fed cells) and group D (SAL fed) had similar levels of IgG ($P > 0.05$), however there were significant differences in responses ($P < 0.01$) between

groups B (OVA fed cells) and E (OVA fed) at d.14 and d.21.

DTH responses

The results in Figure 9.3 show that the response of the OVA fed mice in Group E was 79% suppressed ($P < 0.01$) compared to the SAL fed group D, but this suppression was abrogated by CY (group F). Similarly, mice which had received spleen cells from OVA fed donors had a suppressed DTH response compared to mice which had received cells from SAL fed animals (69% suppression Group A vs. Group B: $P < 0.01$). This suppression was not evident in mice which had received cells from CY pretreated, OVA fed donors (group C). There was no difference in responses between group A (SAL fed cells) and group D (SAL fed), and additionally between group B (OVA fed cells) and group E (OVA fed: $P > 0.05$).

Comments

Very little IgM was present in the serum samples under test, so, in this experiment, the IgM response to OVA did not appear to be suppressed by feeding the antigen before immunization. However, this strategy did result in suppression of the IgG response, and this suppression was unaffected by CY pretreatment. The ability of spleen cells from OVA fed donors to transfer suppression for serum antibody responses did not appear to be a consistent phenomenon, and its validity must be questioned, especially in light of the data contained in the next section.

In contrast, the suppression of DTH responses induced by feeding antigen was fully transferable by giving spleen cells to naive recipients before immunization. CY was able to abrogate both orally induced suppression in vivo and the ability to transfer that suppression, thus reinforcing the idea that oral tolerance for DTH is dependent upon a population of CY-sensitive suppressor cells.

B. CELL TRANSFER Part 2

Experimental protocol

Female BDF_1 mice aged 3 weeks, which were to serve as cell donors, were weaned onto the 4% protein and 24% protein diets, and were maintained on these diets for 2-3 weeks. After this time, the donor animals were fed either 25 mg OVA or 0.2 ml SAL, and were sacrificed one week later. Their spleens were removed, and 1.2×10^8 viable cells were injected i.p. into naive syngeneic recipient mice, aged 8-10 weeks, which had been maintained on the 24% protein diet from weaning. All groups were immunized i.d. into one rear footpad with 100 µg OVA/CFA approximately 4 hrs. later. Serum antibody levels were assessed 2 and 3 weeks after immunization. All groups were skin tested for DTH responses 21 days after immunization by challenging the contralateral footpad with 100 µg OVA/SAL. The footpad was measured immediately before, and 24 hrs after challenge and the difference expressed in mm.

Antibody responses

Transfer of spleen cells from 24% protein OVA fed donor mice did not affect the IgM or IgG response to OVA compared to control mice which had received cells from SAL fed protein sufficient mice (Figs. 9.4, 9.5: $P > 0.05$). Similarly, spleen cells from 4% protein OVA fed mice were also unable to suppress the antibody response ($P > 0.05$ compared to control mice which had received cells from SAL fed 4% protein donors).

DTH responses

The results in Figure 9.6 show that effective suppression of the DTH response was achieved by transferring spleen cells from 24% protein OVA fed mice to a naive recipient before immunization (66% suppression, $P < 0.01$ compared to 24% SAL fed cell recipient mice). In contrast, the diminished response observed in animals which had received cells from 4% protein OVA fed mice was not significantly different from the response of control mice receiving cells from SAL fed protein deficient mice ($P > 0.05$). Furthermore, there was no difference in responses between the two groups which had received cells from OVA fed donors of either dietary group ($P > 0.05$).

Comments

The inability to transfer suppression in this experiment for serum IgM and IgG responses using spleen cells from OVA fed donors is in full agreement with the work of Hanson and

Miller (1982). The reason for the suppression of the responses observed in the last section, therefore, is unclear. As the cell transfer experiments were incidental to the remainder of work in this thesis, this point has not been pursued.

In contrast, oral tolerance for DTH responses was transferable by using spleen cells from protein sufficient OVA fed donors, and this suppression was abrogated by short term protein deprivation. Therefore, it appears that CY and short term protein deprivation adversely affect the same population of cells which transfer tolerance for DTH responses to naive recipients.

Transfer of tolerance with serum from OVA-fed donors

The effect of protein deprivation on the uptake and processing of antigen by the small intestine was investigated in two ways.

a) The quantity of antigen adsorbed from the gut into the circulation one hour after a single feed of antigen was assessed after different periods of dietary protein restriction.

b) The ability of the intestine to process antigen to produce a serum tolerogen for DTH responses was examined after short term protein deprivation.

A. QUANTITY OF CIRCULATING FRAGMENTS OF OVA IN THE SERUM AFTER FEEDING

Experimental protocol

Groups of female BDF_1 mice were weaned onto the 4% or 24% protein diets at 3 weeks of age and were maintained on these diets for 2, 6 or 10 weeks. At these times, the groups of mice received a single oral dose of 25 mg OVA and were exsanguinated 60 mins later from the axillary vein. The serum was separated from the blood samples and stored at $4^{o}C$ before examination. The assay used to measure the level of OVA fragments in the serum was a modified double antibody sandwich ELISA. Each group contained between 5-7 mice.

Results

The concentrations of OVA in the serum of mice fed 25 mg OVA are shown in Figure 9.7. It can be seen that there was a wide scatter of OVA concentrations within each group (e.g. 11.2 - 602.6 ng OVA/ml in 6 week 4% protein group). There was no difference in OVA levels between 4% protein or 24% protein mice after 2 week, 6 week or 10 week periods on the diets ($P > 0.05$). The results suggest that protein deprivation had no overall effect on the uptake of OVA by the gut.

B. A COMPARISON OF THE IMMUNOLOGICAL EFFECTS OF GUT PROCESSED ANTIGEN BY A PROTEIN DEPRIVED OR NORMALLY NOURISHED INTESTINE

Experimental protocol

Groups of female BDF_1 mice were weaned onto the 4% or

24% protein diets at 3 weeks of age and were maintained on these diets for 2 weeks. Animals of both dietary groups were fed either 25 mg OVA or 0.2 ml SAL and were exsanguinated 60 mins later from the axillary vein. Serum from the donor animals of each group was pooled and 0.8 ml injected into each recipient animal. The recipients were female BDF_1 mice which had been maintained on the 24% protein diet for at least 4 weeks from weaning. In addition, a further control group of mice received no serum. All groups were immunized with 100 µg OVA/CFA i.d. into one rear footpad one week after serum transfer, and were bled to measure serum antibody levels 14 and 21 days after immunization. DTH responsiveness was assessed 3 weeks after immunization by challenging the contralateral footpad with 100 µg OVA/SAL and measuring the increment in thickness after 24 hrs. Each serum recipient group contained between 6-8 mice.

Antibody responses

Figures 9.8 and 9.9 show that all groups had identical amounts of IgM and IgG anti-OVA antibodies at d.14 and 21 after immunization. Thus, it appears that serum from OVA-fed donors of both dietary groups had no effect on the humoral response of naive recipients. In addition, the OVA fed serum was unable to prime an animal for subsequent responses to the antigen.

DTH responses

In contrast, the results in Figure 9.10 indicate that serum from both 4% protein and 24% protein OVA fed donors markedly reduced the systemic DTH response to OVA after immunization. 4% protein OVA fed serum recipients exhibited 100% suppression of their DTH response ($P < 0.01$) compared to 4% protein SAL fed serum recipients, while 24% protein OVA fed serum recipients displayed 83% suppression ($P < 0.01$) compared to 24% protein SAL fed serum recipients. The difference in responses between the groups which received OVA fed serum from both 4% and 24% protein donors was not significant ($P > 0.05$) and additionally, there was no difference in responses between the SAL fed serum recipient groups and the group which received no serum ($P > 0.05$).

Comments

The results obtained in this section clearly indicate that protein deprivation did not affect either the quantity or the quality of antigen absorbed by the gut. Thus, the effect which protein deprivation has on the structure of the gut does not appear to lead to abnormal antigen handling. The change in orally induced tolerance for DTH responses caused by short term protein restriction, therefore, is not due to an inability of a malnourished gut to produce a serum tolerogen for DTH after luminal exposure to antigen, but most probably results from the differential effect which protein deprivation has on separate populations of lymphoid cells.

	d.-9	d.-7	d.0
Group	CY?	Fed	Source of cells received
A	-	-	SAL fed
B	-	-	OVA fed
C	-	-	CY/OVA fed
D	0.1 ml SAL	0.2 ml SAL	-
E	0.1 ml SAL	25 mg OVA	-
F	100 mg/kg CY	25 mg OVA	-
G	100 mg/kg CY	0.2 ml SAL	-

All groups immunized d.0 100 µg OVA/CFA

All groups bled d.14,21

All groups DTH test d.21 100 µg OVA.

Table 9.1: Protocol of experiment designed to investigate a) the ability of spleen cells from antigen-fed donors to transfer suppression to naive recipients, and b) the effect of CY pretreatment on oral tolerance and the transfer of suppression.

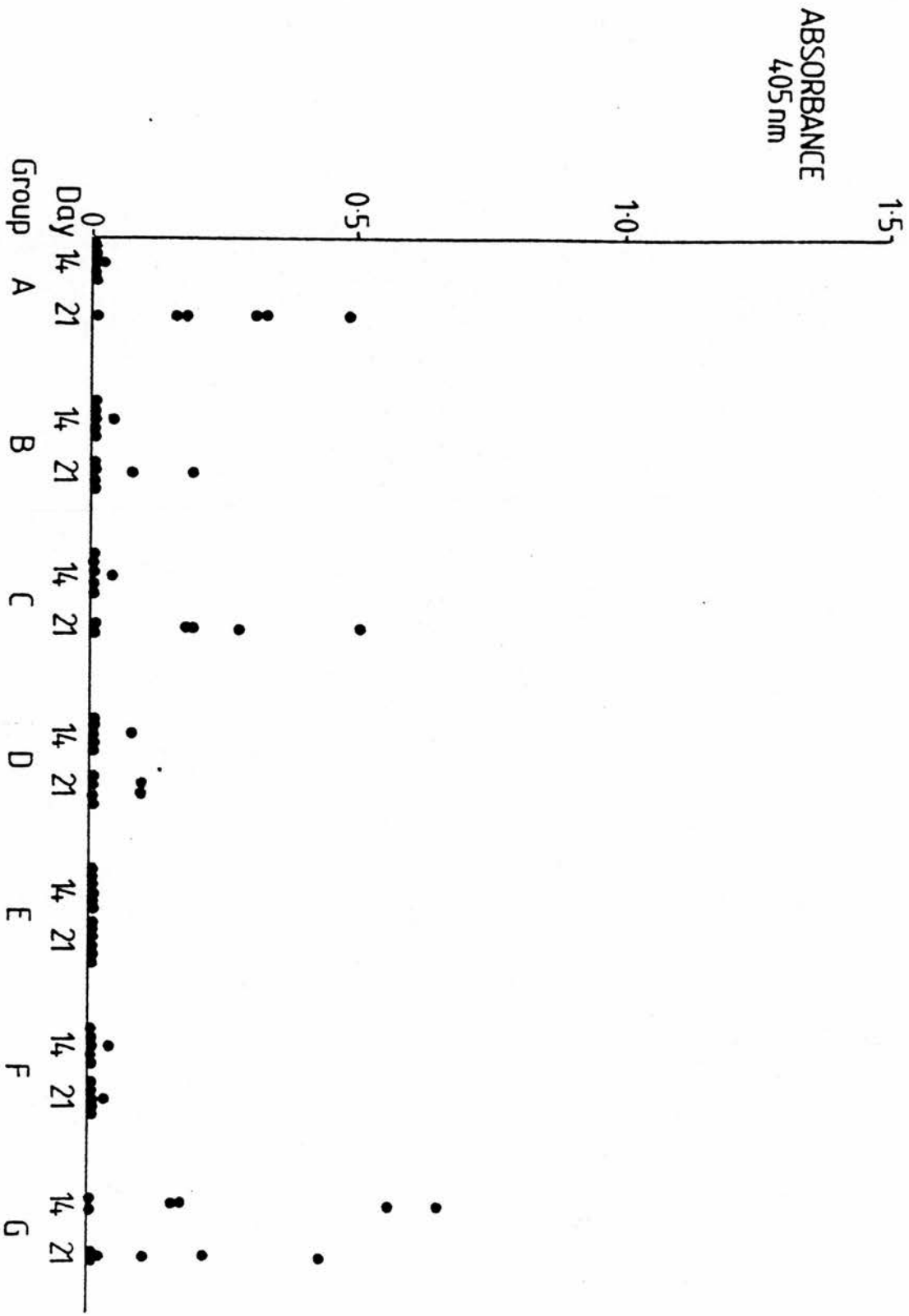

Fig. 9.1: Primary IgM antibody responses of BDF_1 mice - cell transfer section part 1. Antibody levels were assessed d.14 and 21 after immunization. Statistical comparisons are contained within the text (see Table 9.1 for details of the groups).

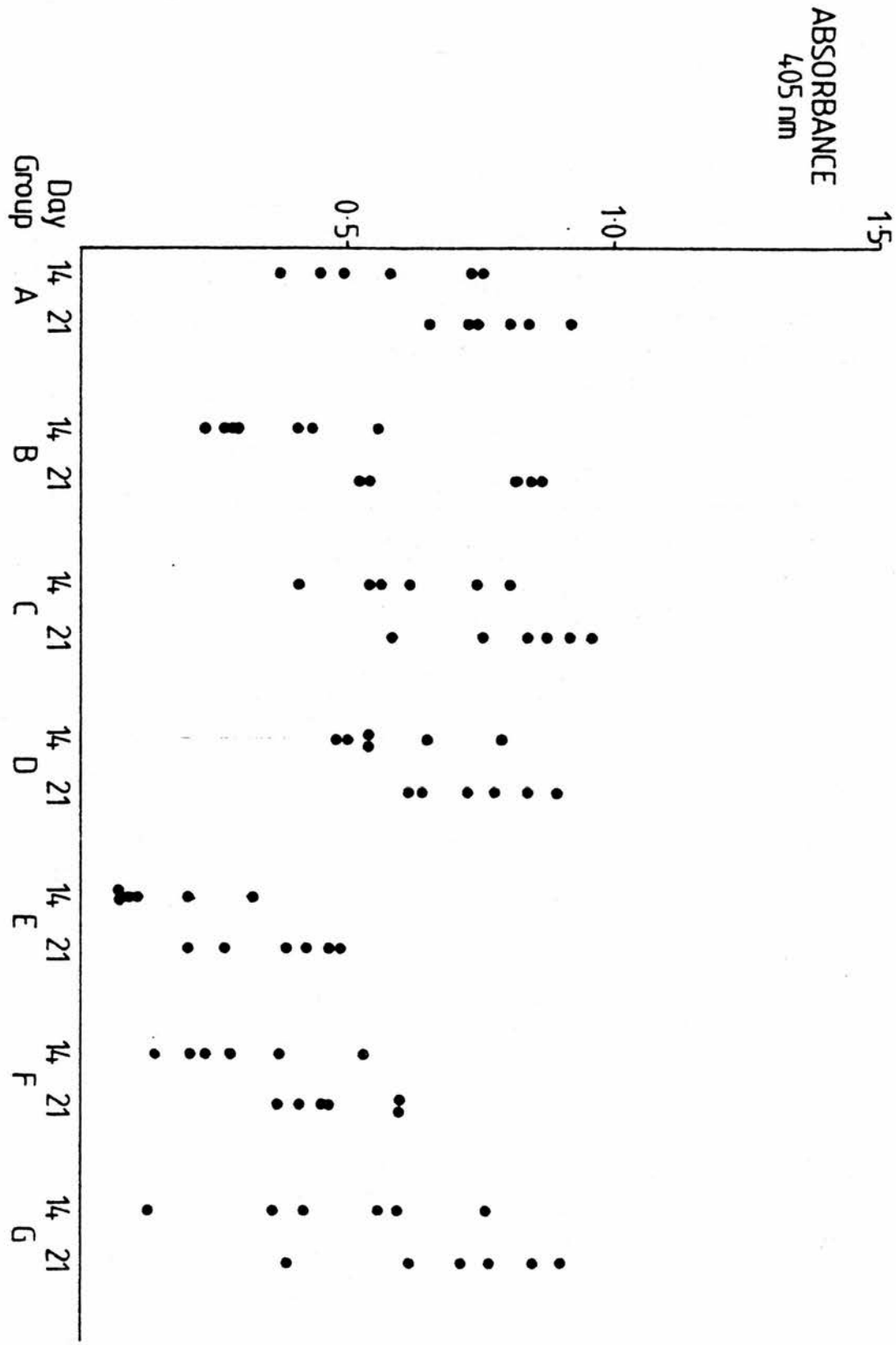

Fig. 9.2: Primary IgG antibody responses of BDF_1 mice - cell transfer section part 1. Antibody levels were assessed d.14 and 21 after immunization. Statistical comparisons are contained within the text. (see Table 9.1 for details of the groups).

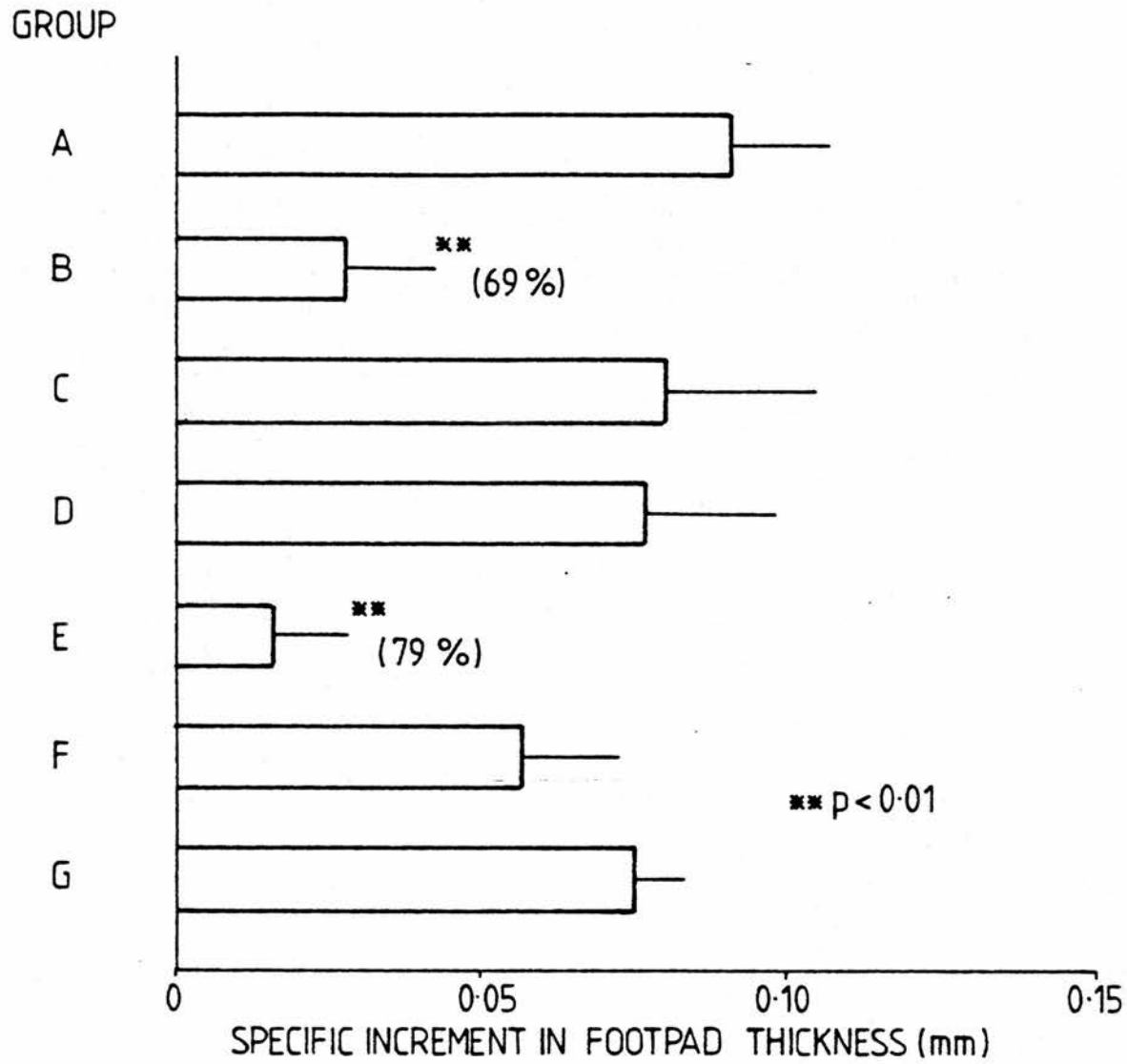

Fig. 9.3: DTH responses of BDF_1 mice - cell transfer section part 1. DTH was assessed d.21 after immunization. Bars represent the mean + 1 s.d. of 5-8 mice. The figures for % suppression compare groups A vs. B and groups D vs. E (see Table 9.1 for details of groups).

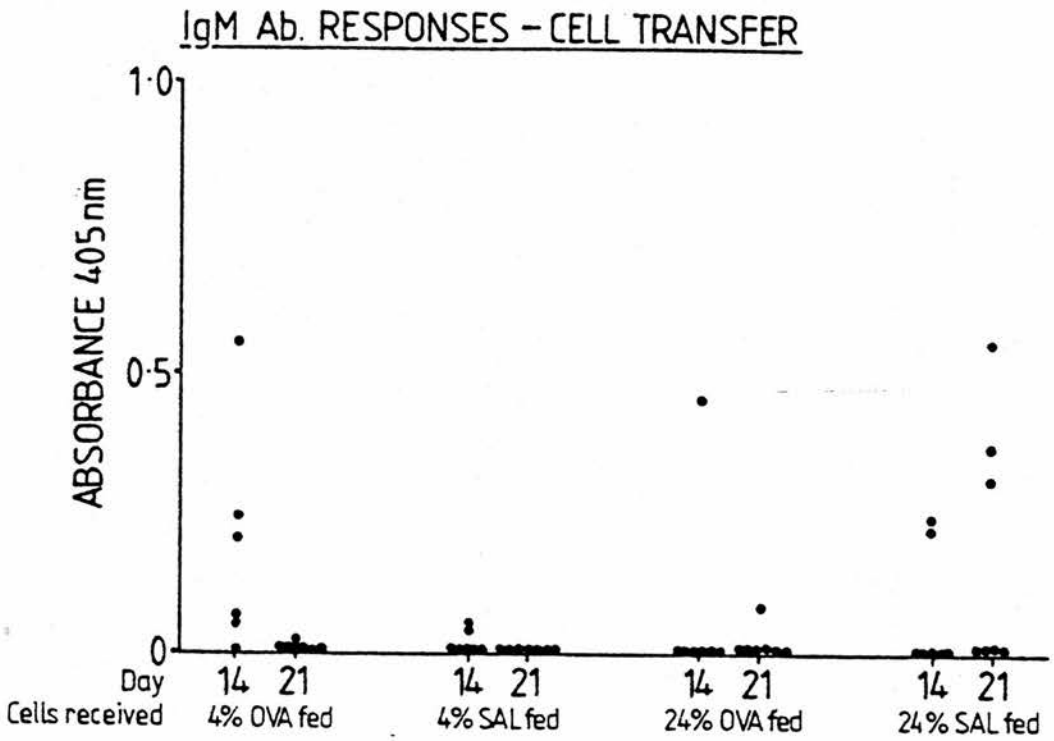

Fig. 9.4: Primary IgM antibody responses of BDF_1 mice - cell transfer section part 2. All groups were immunized approximately 4 hrs. after transfer of cells and antibody levels were assessed 14 and 21 d. later. Statistical comparisons are contained within the text.

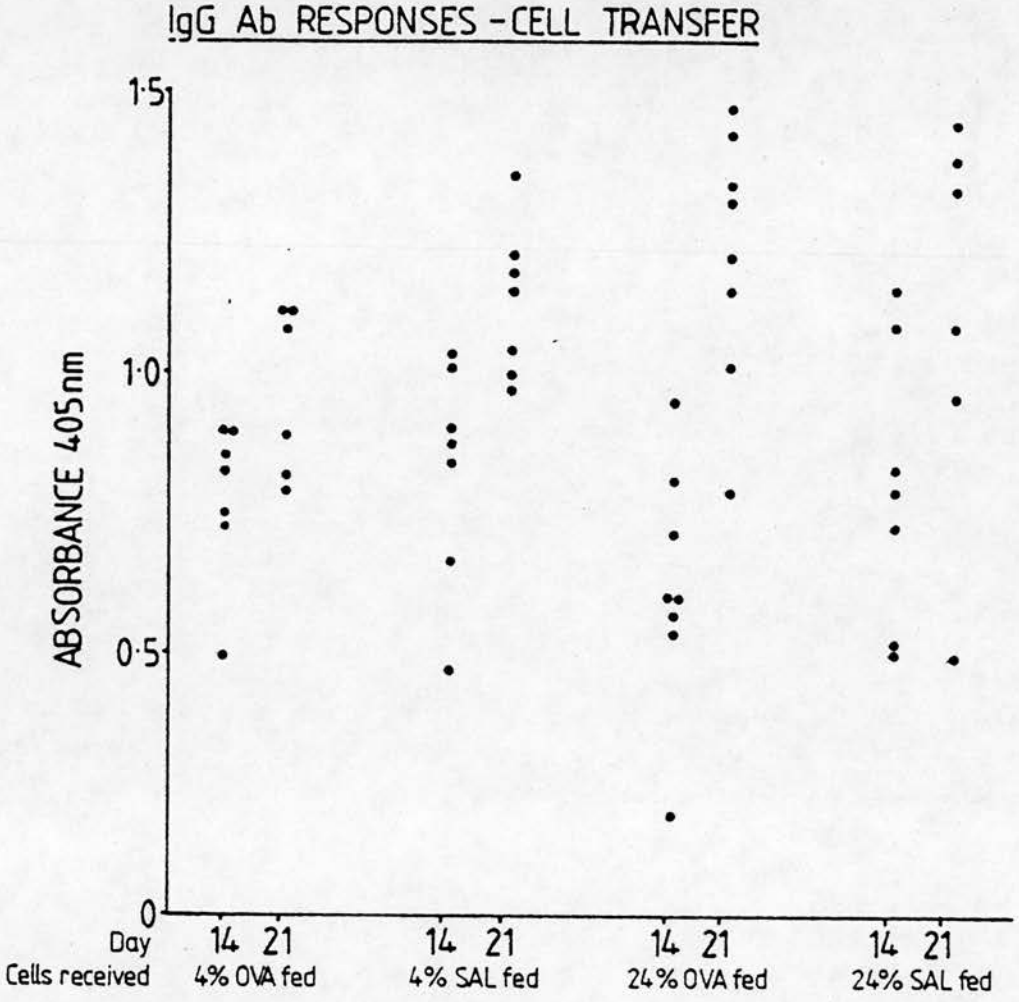

Fig. 9.5: Primary IgG antibody responses of BDF_1 mice - cell transfer section part 2. All groups were immunized approximately 4 hrs. after transfer of cells and antibody levels were assessed 14 and 21 d. later. Statistical comparisons are contained within the text.

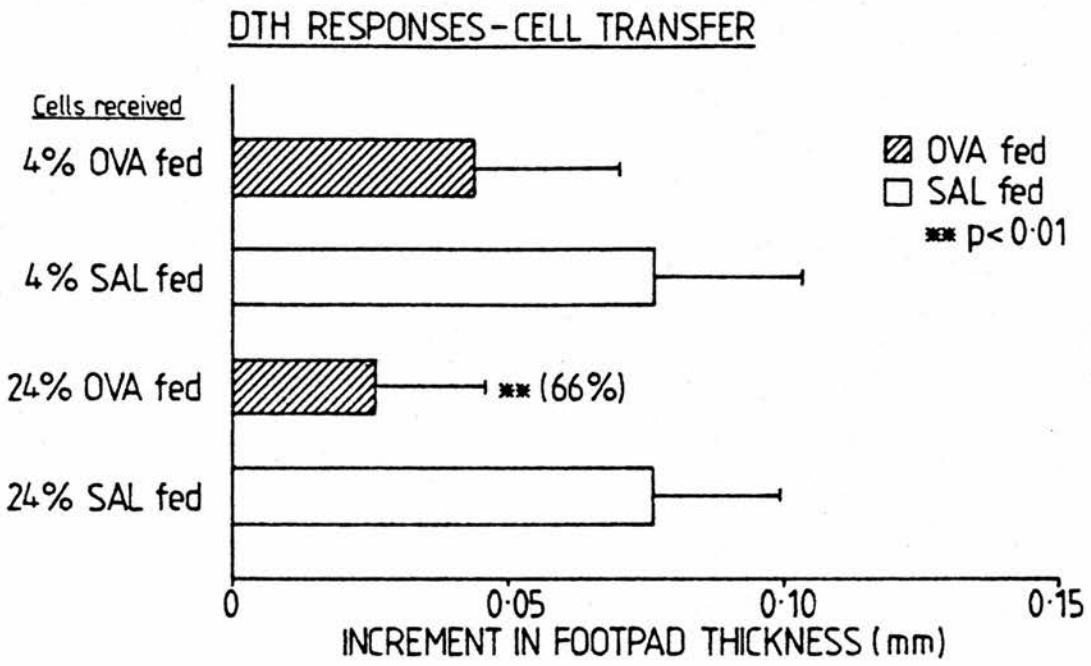

Fig. 9.6: DTH responses of BDF_1 mice - cell transfer section part 2. All groups were immunized approximately 4 hrs. after transfer of cells and DTH was assessed d.21 after immunization. Bars represent the mean + 1 s.d. of 6-8 mice. The responses of OVA fed cell recipients are compared to those of SAL fed cell recipients, and the figure for % suppression in brackets is for 24% protein maintained donors.

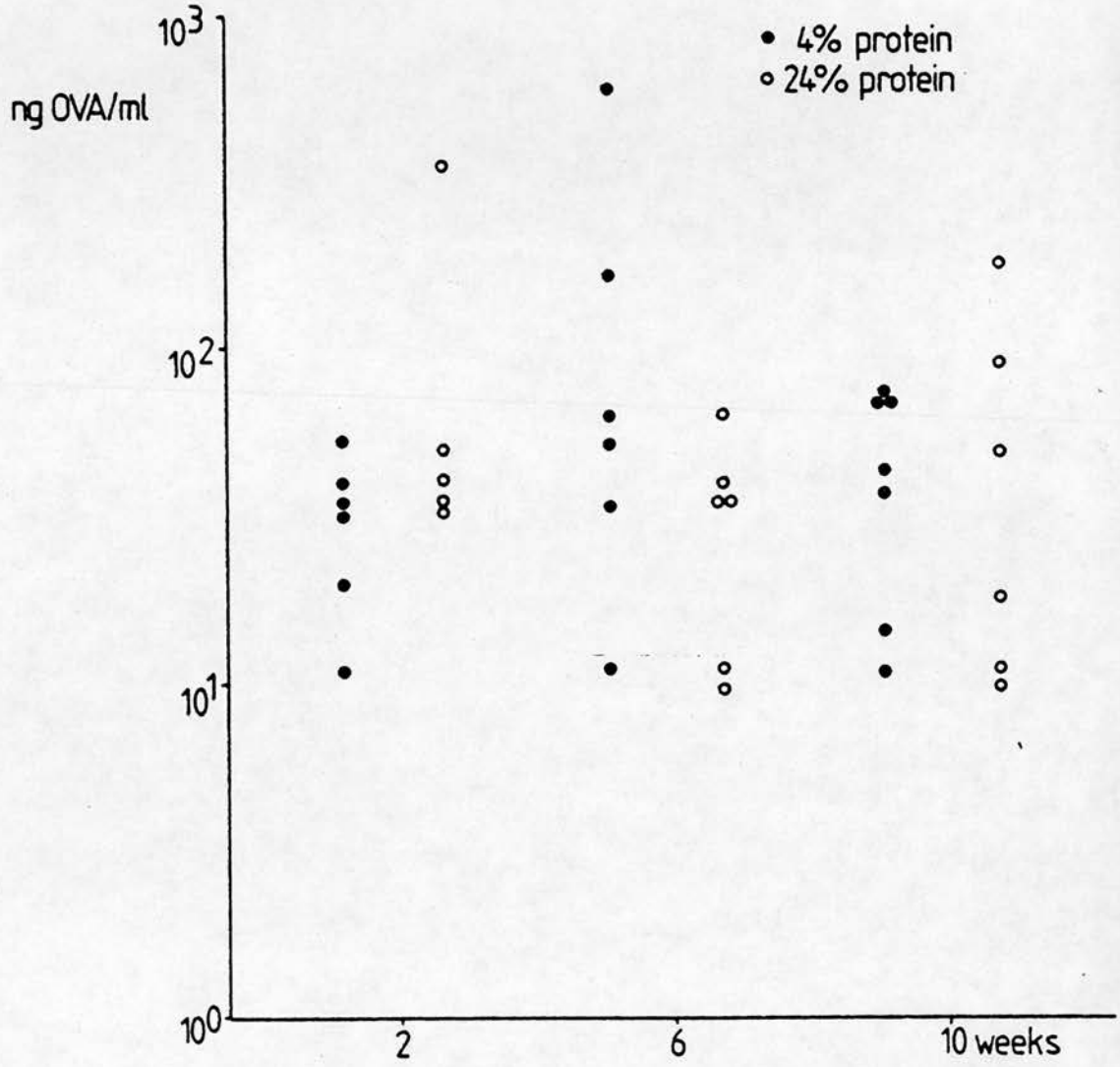

Fig. 9.7: Effect of protein deprivation on the transport of OVA from the gut lumen to the blood. Groups of mice were maintained on diets containing 4% or 24% protein for 2,6 and 10 weeks from weaning. Serum concentrations of OVA were measured by ELISA one hour after feeding. Statistical comparisons are contained within the text.

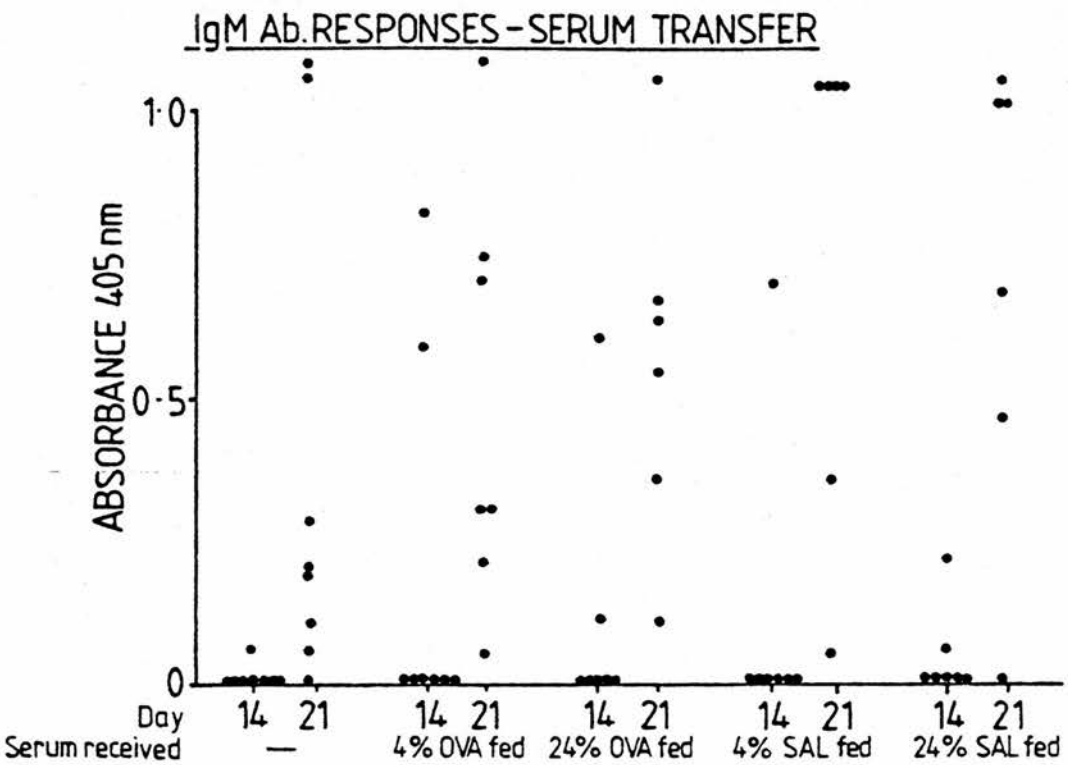

Fig. 9.8: Primary IgM antibody responses of BDF_1 mice - serum transfer. Control group received no serum. All groups were immunized 1 week after serum transfer and antibody levels were assessed 14 and 21 d. later. Statistical comparisons are contained within the text.

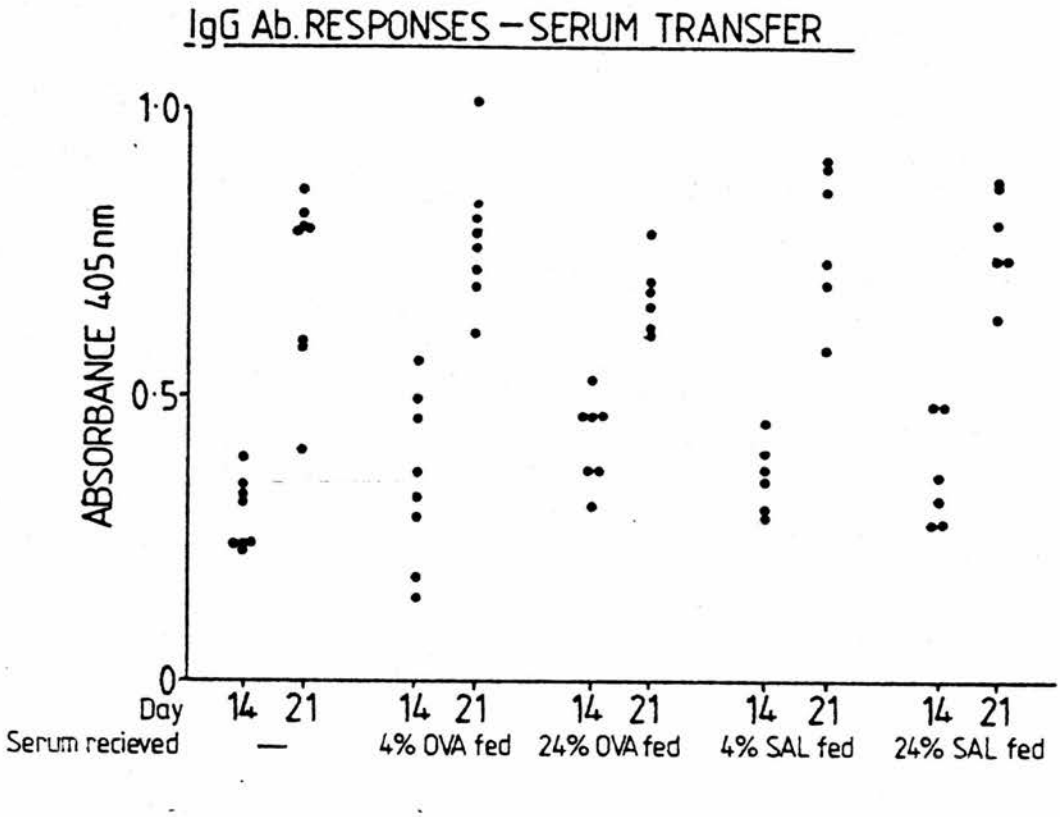

Fig. 9.9: Primary IgG antibody responses of BDF_1 mice – serum transfer. Control group received no serum. All groups were immunized 1 week after serum transfer, and antibody levels were assessed 14 and 21 d. later. Statistical comparisons are contained within the text.

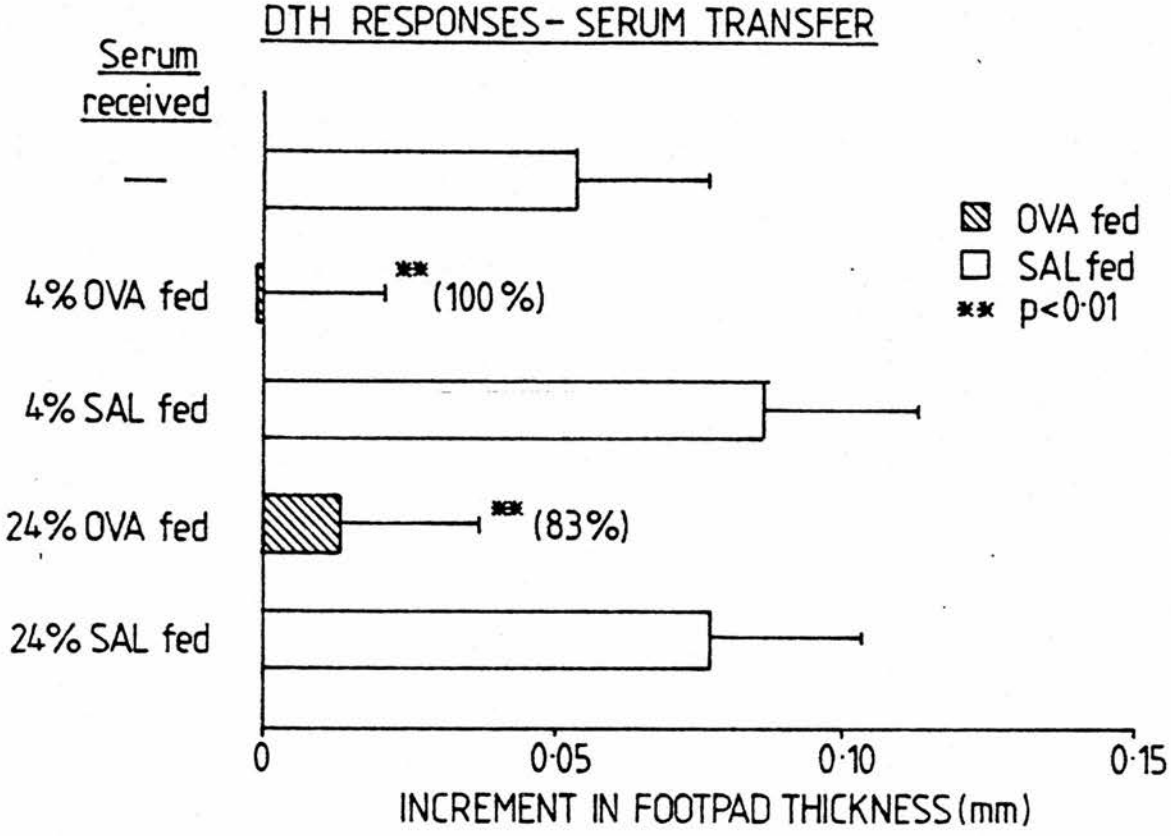

Fig. 9.10: DTH responses of BDF_1 mice - serum transfer. Control group received no serum. All groups were immunized 1 week after serum transfer and DTH was assessed 21 d. after immunization. Bars represent the mean + 1 s.d. of 6-8 mice. The figures for % suppression of responses of OVA fed serum recipients vs. SAL fed serum recipients are given in brackets.

CHAPTER 10

THE EFFECT OF PROTEIN DEPRIVATION ON THE EXPRESSION OF LOCAL CMI RESPONSES IN THE GUT

Introduction to experiments

Previous work from this laboratory has demonstrated that in conditions where oral tolerance for systemic DTH responses is impaired, an active local CMI response can be induced in the small intestine (Mowat and Ferguson, 1981(a)). This response can be measured indirectly by examining changes in a) the production rate of cells within the intestinal crypts b) the crypt depth and c) IEL count. Mucosal CMI responses have been observed after tolerance has been abrogated in a number of ways, e.g. after treatment with CY, oestradiol and MDP (see Chapter 2). Additionally, damaging CMI responses can also occur in the intestinal mucosa of F_1 animals undergoing a mild GvHR following injection of parental lymphocytes.

As short term protein deprivation can inhibit orally induced tolerance for DTH responses, it is predicted that these mice would be uniquely susceptible to the damaging consequences of a mucosal CMI reaction. The aim of this section, therefore, was to examine the effect of protein deprivation on the expression of intestinal CMI. This was accomplished in two ways:-

a) by inducing a limited GvHR in protein deprived F_1 mice

b) after continuous oral challenge of protein deprived mice with antigen.

GvHR IN PROTEIN DEPRIVED BDF_1 MICE

Experimental protocol

Groups of female BDF_1 mice ($H\text{-}2^{b/d}$) aged 3 weeks were weaned onto 4% and 24% protein diets, and were maintained on these diets for the duration of the experiment. Three weeks after initiation of the diets, groups B (24% protein) and D (4% protein) each received 6×10^7 spleen cells i.p. from C57BL/6J ($H\text{-}2^{b/b}$) female donors aged 6-8 weeks. Control groups A (24% protein) and C (4% protein) received 0.2 ml RPMI 1640 i.p. at this time. Eight and fourteen days after induction of GvHR, mice from each group were sacrificed, and their spleens were removed and weighed to assess splenomegaly. In addition, samples of jejunum were removed for histological and microdissection analysis. Each group contained between 6-8 mice.

Development of the GvHR

All cell recipient animals remained healthy throughout the experiment. Group B had spleen indices of 1.7 at d.8 and 2.1 at d.14 indicating substantial splenic hypertrophy at these times (see Table 10.1). Unfortunately, insufficient numbers of mice in group C meant that the spleen index could not be assessed in group D at d.8. However at d.14, group D showed significant splenomegaly (spleen index 1.7).

Mucosal morphology during GvHR

The results in Table 10.1 indicate that in the GvHR group B, there was a significant increase in IEL count at d.8 compared to group A ($P < 0.01$). However, by d.14, there was no difference in numbers between the two groups ($P > 0.05$). In the 4% protein groups, IEL counts were similar in the control group C and experimental group D at d.14 ($P > 0.05$).

Microdissection analysis of jejunal samples revealed that the villous height was unchanged in group B at d.8 and d.14 compared to group A ($P > 0.05$). Crypt depth, however, was increased at both time points in the GvHR group B ($P < 0.01$). CCPR, although slightly raised at d.8 was normal at both days compared to group A ($P > 0.05$).

As expected, measurements of crypt depth were decreased in the 4% protein groups compared to the equivalent 24% protein groups. However, in this case, there was no difference in crypt depths between the control group C and the cell recipient group D at day 14 ($P > 0.05$). Similarly, both villous height and CCPR in group D were normal at d.14 ($P > 0.05$).

Comments

The results from this experiment have confirmed the data of other workers which show that a GvHR can induce changes in the gut mucosa resulting from a local CMI reaction.

The development of the GvHR was assessed by following changes in the weight of the spleen caused by the proliferation

of cells occurring within this organ. In the 24% protein experimental group, the weight of the spleen was increased at both times after GvHR induction. In the low protein environment however, the extent of this splenomegaly was reduced. This suggests that cellular proliferation within the spleen, although still apparent, was much impaired.

The most sensitive parameter of a mucosal CMI response in this work was an increase in crypt depth and this occurred in the 24% protein GvHR group at both days examined. Of the other indicators of CMI, IEL counts were increased at d.8 but not at d.14, and the CCPR, although slightly raised at d.8, was not significantly different from control values. The CMI reaction in the gut appeared greater at d.8 than it was at d.14 after GvHR induction. In contrast, none of the changes associated with a mucosal CMI response were present in the experimental 4% protein group at d.14. Furthermore, the crypt length, CCPR and IEL counts did not appear to be inordinately raised in this group at d.8.

These results demonstrate that the GvHR induced mucosal CMI reaction is restricted in 4% protein animals and they further indicate the importance of the protein deprived environment in preventing normal expression of responses.

INDUCTION OF MUCOSAL CMI BY FEEDING AND CHALLENGING WITH OVA

The hypothesis which was tested in this section was that short term protein deprivation, by eliminating suppressor cells, will allow for the induction of local CMI in the gut

after feeding antigen. The ability of oral immunization to prime for a systemic DTH response in these mice was also examined in parallel.

Experimental protocol

Groups of female BDF_1 mice aged 3 weeks were weaned onto diets containing 4% and 24% protein, and were continued on these diets for the duration of the experiment. Two weeks after initiation of the diets, animals were fed 2 mg OVA or 0.2 ml SAL according to the group protocol (Table 10.2). Group 8 received 100 mg/kg CY i.p. 2 days previously. 21 days after feeding, experimental groups were challenged over a period of 10 days with 100 µg OVA/day/mouse in their drinking water. Control groups received 100 µg HSA/day/mouse in tap water alone. At the end of the challenge period, the mice were killed, and samples of jejunum were removed for histological and microdissection analysis. Groups 2, 6 and 8 were tested for systemic DTH by footpad swelling response 21 days after oral immunization and on completion of the 10 day oral challenge. The footpad was measured immediately before and 24 hrs after challenge with 100 µg OVA/SAL and the difference expressed in mm. In addition, control mice in each group were challenged with 0.05 ml SAL. Every group in the experiment contained between 6-8 mice.

Systemic DTH responses

The DTH responses of orally immunized mice to OVA were compared to the responses of control mice challenged with SAL, and the results shown in Table 10.3. At d.21 after oral immunization, there was no difference in responses between the OVA challenged and the SAL challenged animals of any group ($p > 0.05$). Similarly, after the 10 d. oral challenge protocol (d.31), no DTH response was elicited in any of the groups compared to controls ($P > 0.05$).

Mucosal morphology

IEL counts from the groups are shown in Figure 10.1. In this case, the 4% protein groups had lower IEL numbers when compared to the equivalent 24% protein group (groups 1 vs. 5: 2 vs. 6: and 3 vs. 7: $P < 0.01$). There was no difference in counts between the experimental 4% protein group 2 and the control 4% protein groups 1 and 3 ($p > 0.05$). It was strange to note, however, that the CY treated, OVA fed and challenged group 8 had identical counts of IELs compared to the control group 6, which did not receive CY ($P > 0.05$).

Short term protein deprivation significantly reduced the crypt depth in groups 1-4, but did not affect the CCPR compared to the appropriate control group 5-7 (Table 10.4). Villous heights were decreased in the protein deprived groups, but the differences were significant only in groups 1 vs. 5 ($P < 0.05$) and groups 3 vs. 7 ($P < 0.01$). In 24% protein mice given CY before feeding OVA (group 8), crypt depth was

increased ($P < 0.01$ compared to group 6). However, although CCPR was slightly elevated in this group, this difference was not significant ($P > 0.05$ compared to group 6). None of the mucosal changes associated with a CMI response in the gut were observed in the experimental 4% protein group 2. The values for both the crypt depth and the CCPR were normal compared to groups 1 and 3 ($P > 0.05$).

Comments

The results of the experiments obtained here are consistent with the finding from the previous section that protein deprivation can prevent the expression of local CMI in the gut.

This work also confirms the observations of others that pretreatment of a protein sufficient host with CY 2 days before feeding OVA, can allow for the development of mucosal changes consistent with a CMI reaction. Oral immunization, however, did not significantly prime an animal for a subsequent systemic DTH response.

In common with the local reaction induced by a GvHR, an increase in crypt depth appeared to be the most consistent feature of the response, with the increase in IEL count or CCPR not apparent at the time when the sample of jejunum was taken. It is possible that these other parameters of a mucosal CMI response may be present in this model if tissue is taken at other times.

	Relative spleen weight (mg/10 g body wt.)	Spleen index	Villous height (μm)	Crypt depth (μm)	CCPR (cells/ hr)	IEL number (cells/100 e.c.)
Group A d.8	48.1 $\pm$3.3	1.7	778.5 $\pm$20.1	113.4 $\pm$ 2.5	12.8	13.7 $\pm$0.8
Group B d.8	83.7 $\pm$13.3		806.5 $\pm$70.9	134.3 $\pm$ 4.0	15.3	19.5 $\pm$1.0
Group A d.14	49.2 $\pm$ 4.7	2.1	751.4 $\pm$ 22.9	112.9 $\pm$ 3.5	12.3	15.8 $\pm$1.0
Group B d.14	102.1 $\pm$20.6		702.6 $\pm$ 48.3	130.5 $\pm$ 6.7	12.7	16.3 $\pm$0.7
Group C d.8	N.D.	-	N.D.	N.D.	N.D.	N.D.
Group D d.8	45.0 $\pm$ 6.7		619.4 $\pm$30.1	96.3 $\pm$2.6	10.1	13.8 $\pm$ 1.2
Group C d.14	31.7 $\pm$ 6.2	1.7	642.7 $\pm$ 97.5	94.8 $\pm$3.1	9.2	14.0 $\pm$ 2.1
Group D d.14	54.5 $\pm$15.6		668.1 $\pm$ 63.7	97.0 $\pm$4.8	9.9	15.9 $\pm$ 1.6

N.D. = not determined.

Table 10.1: GvHR induced in 4% and 24% protein BDF_1 mice by injection of normal parental spleen cells. Spleen and intestinal samples were taken 8 and 14 d. after initiation of GvHR. The figures represent the mean $\pm$1 s.d.of 6-8 mice. Group descriptions and statistical comparisons are contained within text. Spleen index calculated as described in Chapter 3.

Group	Diet	CY?	Fed	Challenged	DTH Skintest?
1	4%	-	OVA	H_2O	-
2	4%	-	OVA	OVA	+
3	4%	-	SAL	OVA	-
4	4%	-	OVA	HSA	-
5	24%	-	OVA	H_2O	-
6	24%	-	OVA	OVA	+
7	24%	-	SAL	OVA	-
8	24%	CY	OVA	OVA	+

Table 10.2: Protocol describing the treatment of groups in the experiment studying the effect of protein deprivation on mucosal CMI.

Group	Days after 1st oral immunization	DTH response (mm) after challenging with:-		P
		0.05 ml SAL (n=2)	100 µg OVA (n=4)	
2	21	0.055 ± 0.007	0.054 ± 0.038	n.s.
6	21	0.055 ± 0.021	0.013 ± 0.040	n.s.
8	21	0.045 ± 0.064	0.045 ± 0.031	n.s.
2	31	0.020	0.040 ± 0.020	n.s.
6	31	0.005 ± 0.007	-0.033 ± 0.015	n.s.
8	31	0.020	-0.008 ± 0.038	n.s.

Table 10.3: DTH responses of BDF_1 mice following oral immunization with 2 mg OVA. DTH was assessed by footpad swelling response 21 and 31 d. (the latter following completion of 10 d. OVA challenge protocol) after first immunization. The figures represent the mean ± 1 s.d. of 6-8 mice. (see Table 10.2 for group protocol).

Group	Villous height (μm)	Crypt depth (μm)	CCPR (cells/hr)
1	779.4 ± 39.3	109.0 ± 7.8	11.4
2	785.7 ± 28.6	114.3 ± 5.6	10.0
3	764.2 ± 66.8	106.6 ± 8.2	12.4
4	814.3 ± 85.1	111.2 ± 8.3	9.0
5	853.3 ± 58.8	124.3 ± 7.2	13.1
6	826.0 ± 35.6	124.5 ± 5.4	13.3
7	874.5 ± 30.0	124.4 ± 4.2	11.0
8	830.5 ± 34.0	134.2 ± 3.4	15.0

Table 10.4: Measurements of intestinal structure in BDF_1 mice following completion of 10 d. OVA challenge. The figures represent the mean ±1 s.d.of 6-8 mice, and statistical comparisons are contained within the text. (See Table 10.2 for group protocol).

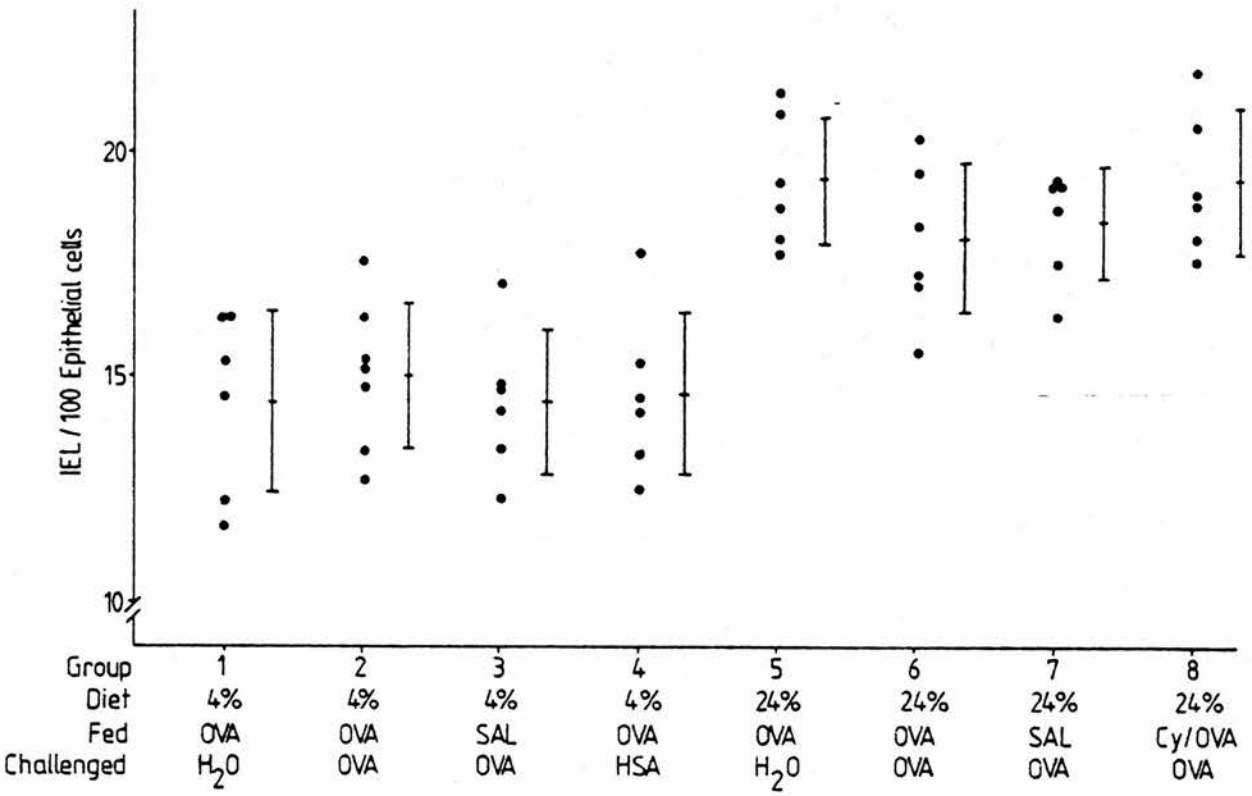

Fig. 10.1: IEL counts from jejunum of BDF_1 mice following completion of the 10 d. oral challenge with OVA. Bars represent mean $\pm$ 1 s.d. of 6-8 mice and comparisons between the groups are given in the text. (See Table 10.2 for group protocol).

CHAPTER 11

GENERAL DISCUSSION

Introduction

The subject under study in this thesis has been the effect of protein deprivation on intestinal immune function. Having first established a suitable mouse model of this condition, I examined various physiological and immunological parameters of these animals.

Protein deprivation reduced the size of intestinal villi and crypts, although the gut retained a normal structure. Lymphoid tissue was severely affected, with all the organs studied, showing varying degrees of atrophy. T lymphocyte function, as assessed in in vivo tests, was impaired in protein deprived animals, however, when these cells were removed from this restrictive environment, as in the GvHR assay, their function appeared normal.

A most interesting observation was made when the influence of protein deprivation on orally induced tolerance for systemic immune responses was investigated. While the extent of suppression for antibody responses was increased, the degree of suppression for DTH responses was decreased. Further investigations into the disparate effect of protein deprivation on the humoral and cell mediated limbs of and tolerance were accomplished using serum and cell transfer protocols.

When oral tolerance for systemic DTH responses is impaired, a local CMI response in the gut can be induced by feeding antigen (Mowat, 1981). It was originally envisaged, therefore, that protein deprived mice would be

uniquely susceptible to mucosal damage caused by a DTH response to the fed antigen. This situation, however, did not happen. Furthermore, the mucosal CMI response which normally occurs during a GvHR was also prevented.

As the establishment of the model was initially important, I shall first discuss this aspect of my work, followed by the basic observations of immune structure and function performed with the model. Thereafter, the effect of protein deprivation on oral tolerance, with emphasis on the mechanisms involved, will be considered. Finally, I shall discuss the work relating to the lack of damaging immune reactions in the intestine of the protein deprived animals.

Characteristics of the model of protein deprivation

In the model of protein deprivation in these experiments, I decided to use a level of 4% protein in the restricted diet. This decision was reached after examining previous literature using animal models of protein deprivation, and was within the range (2% ➟ 8%) of dietary protein associated with most of these models. The normal diet contained 24% protein, which was also within the range (18% ➟ 30%) used for the positive control animals in published work.

In a few experiments (from Chapters 7 and 9), mice were used which had been maintained on the standard laboratory diet (18.5% protein). The size and weight of these mice was similar to that of age-matched 24% protein mice, and I felt justified in using them as recipient animals for 4% or 24% protein parental cells in the GvHR assay and in

the first cell transfer experiment. As far as possible, however, the control groups in the other experiments were maintained on the 24% protein diet.

Only one level of dietary protein restriction was used throughout the thesis. I felt that it would be more productive to concentrate solely on one model of protein deprivation and examine this closely, rather than to investigate several levels of restriction. Multiple groups of protein deprived mice might have presented additional problems, such as escape and penetration of mice from one protein deprived dietary group into the cages of another protein deprived group. Furthermore, purchase of the diet in reasonable quantities was relatively expensive, and I did not think the extra cost justified within the scope of this thesis. In certain circumstances however (as will be discussed later), an additional level of protein deprivation (e.g. 8%) might have proved useful to investigate.

Mineral deficiencies can have particularly significant effects on immune function. For instance, deficiency of zinc in the diet can produce progressive thymic involution and a loss of T lymphocyte responses in mice and rats (Good, West and Fernandes, 1980). The concentration of zinc in the mineral premix used in the 4% protein diet was doubled to prevent this (see Table 3.2). Concentrations of other dietary constituents were also increased in the 4% protein diet. In general, I hoped to ensure that by providing increased quantities of vitamins and minerals, only protein

deficiency would occur in these mice. Additionally, the 4% and 24% protein diets were made isocaloric in order to prevent calorie deficiencies. With respect to this point, published reports have shown that protein deprived mice and rats ate more food per gram of body weight compared to control animals (Bell et al., 1976(a): Barry and Pierce, 1979). The authors have suggested that in these cases, the protein deprived diets were limiting only for protein.

Pair feeding experiments were not attempted during the course of this work. The main reason behind this decision was the nature of the work involved. The project was approached from an immunological and not a nutritional viewpoint. While strict nutritional research requires pair feeding trials with small groups of animals, the experiments in this thesis used many groups of animals, with large numbers of mice per group. Therefore, I did not consider it realistic to pair feed animals. Furthermore, there was a limited time available to complete the project. Pair feeding trials would have occupied a considerable amount of time and would necessarily have restricted the numbers of experiments which would have been completed.

Protein deprivation had an immediate and severe effect on both the weight and physical appearance of BDF_1 mice. These mice failed to grow in size compared to 24% protein mice, and the texture of the coat was notably changed. It was only after approximately four weeks deprivation that the mice registered slight increases in weight. It is interesting to note that the effects of short term restriction

(e.g. 2 weeks) appeared more severe than those observed after longer periods of restriction (e.g. 8 weeks). This suggests that, to a certain extent, the mice adapted to cope with a reduced protein intake. This ability to adapt is further demonstrated by the group of mice which have been maintained on the 4% protein diet for over one year, and it also indicates that the diet is sufficient to survive on, if not sufficient for normal growth patterns. This feature of adaptation after prolonged periods of restriction is a common one, and will be repeated throughout the discussion.

The characteristic physical appearance and failure to gain weight in short term protein deprived BDF_1 mice was not observed when BALB/c mice were subjected to this type of nutritional insult. In this case, these mice gained weight immediately and there was no change in coat texture. The low protein BALB/c mice, however, were smaller than age matched controls. The reasons for the differences between the two strains in their ability to cope with protein deprivation are not clear, but may involve differences in basal metabolic rates and/or the ability to utilise the reduced protein content that is available in the diet.

As mentioned before, protein deprived mice, even members of the same litter, often showed a great deal of variation in size and appearance. This was probably related to differences in food consumption between mice, a factor which was exacerbated by having large numbers of mice per cage as in the initial experiments. By reducing the numbers

housed per cage, the variation in size and appearance of the protein deprived mice (and also the death rate - Chapter 4) was substantially decreased.

Total serum protein content of deprived mice was lower than control mice at all points examined. This observation is consistent with data describing this parameter both in animal models of protein deprivation (Rodrigues, De Camargo, Coelho, Montenegro, Angeleli and Burini, 1985) and also in malnourished human populations (Searcy, 1969). Serum albumin concentration, however, was not such a reliable indicator of protein deprivation in this model, with the concentration at 6 weeks only being depressed in comparison to control mice.

The structure of intestinal and systemic lymphoid tissue

Examination of lymphoid tissue in protein deprived mice provided some interesting observations on the environments in which immune responses occur *in vivo*. Of all the organs and tissues studied, only liver was reduced in proportion to the reduction in body size as a whole. The remaining structures all showed a disproportionate reduction in size and cellularity, which was most severe in the thymus, followed by the spleen, and least severe in the lymph nodes.

These findings agree completely with observations made by Bell and colleagues on their model of protein deprivation (Bell et al., 1976(a)). However, one major difference between the models does exist. In the work

described here, the effects of protein deprivation on lymphoid tissue appeared more severe after 2 weeks than after 6 weeks. This was true most noticeably for the thymus, and to a lesser extent for the spleen and Peyer's patches. While the reason for this recovery effect is not entirely clear, it may be partly related to the increased levels of serum cortisol present in protein deprived animals (Aschkenasy et al., 1966). The lympholytic effects of corticosteroid hormones are well known, with thymus cortical cells being particularly susceptible (Cohen, Fishback and Claman, 1970). Stress induced by initial periods of protein deprivation may have increased adrenal production of this hormone. However, with continuing time on the 4% protein diet, the animal adapted to cope with the reduced protein intake, and production would have decreased. In retrospect, it may have proved interesting to monitor levels of this hormone during the course of a protein deprivation experiment.

Differences in mouse strain used must also be considered when comparing results between the models of protein deprivation. In my work, I have used only two strains of mice. Even within this limited scope however, differences in physical appearance and immune responses between them were observed (Chapters 4 and 7). Tissue from protein deprived BALB/c mice was not taken for histological examination so it was impossible to tell whether the lymphoid organs of these mice were similarly affected.

The depletion of cells within the thymic cortex was a striking feature of 2 weeks protein deprivation, and contrasted with the relative resistance of the medullary thymocytes. However, prediction of T lymphocyte function in deprived animals from these observations alone is complicated by the controversies which exist about T lymphocyte differentiation within this organ. For instance, cortical thymocytes make up approximately 85% of the total cell population of the thymus, are phenotypically immature and cannot be induced to function either in vivo or in vitro (Scollay, 1982, Scollay, 1983). On the other hand, medullary thymocytes are mature in phenotype, and can be induced to function (Chen, Scollay and Shortman, 1982). It would appear that medullary cells are the precursors of peripheral T cells, waiting their turn for export into the circulation. However, when a monoclonal antibody (MEL-14) recognizing a molecule involved in lymphocyte recirculation (Gallatin, Weissman and Butcher, 1983) is used to stain the thymus, the only positive cells are contained within the cortex (Scollay, 1983). This clearly implies that a population of cortical cells may be exported from the thymus to the periphery. With respect to the above data on protein deprived mice, therefore, it is unwise to predict or confirm a defect in T lymphocyte function in these animals merely from the histological observation of altered thymic structure.

The effect of protein deprivation on the internal structure of the spleen provided some interesting points. The PALS region around the central arteriole was well maintained after 2 weeks deprivation, thus confirming the persistence of a population of long lived recirculating T cells in 4% protein mice suggested by others (Bell et al., 1976(a); Malavé et al., 1980). However, after 6 weeks, this area showed a loss of these cells. With prolonged periods of deprivation therefore, even long lived T cells were depleted from the animal. Alternatively, migration and recirculation of these cells may be impaired.

The loss of definition of distinct microenvironments in protein deprived animals was especially apparent within the spleen of 2 week deprived mice (Fig. 6.2(b)). While the mechanisms responsible for the arrangement of specific T and B areas within lymphoid tissue remain unclear, the importance of accessory cells in determining migration patterns within this tissue has been suggested (Haston, 1979). Additionally, interaction of lymphocytes with supporting stroma within specific microenvironments is being increasingly defined (Butcher, 1983). Although it is possible that a defect in migratory properties of the deprived cells themselves may underlie this loss of definition, it is also possible that protein deprivation can affect the ability of accessory cells and stroma of distinct microenvironments to influence cell localization. The solution to this problem awaits a better understanding of the process by which cells

arrange themselves into specific areas. Furthermore, the disordered internal organization within the spleen may contribute to the depressed immune responses occurring after short term deprivation, by impairing the interactions and cooperation between lymphocytes and antigen presenting cells necessary for the induction of immune responses.

A similar situation was observed within the Peyer's patches, where 2 weeks protein deprivation was shown to alter the internal organization of this tissue (Fig. 6.3(b)) compared to that of controls. The germinal centre of the patch was small and poorly defined. This fact, together with the proposed defect in lymphoblast localization within the small intestine (McDermott et al., 1982) predicts that the intestinal sIgA response to antigen in these mice will be impaired. This has already been demonstrated with the secretory antibody response to cholera toxin in a rat model of protein deprivation (Barry and Pierce, 1979) and is the subject of current studies in this laboratory. It will be of interest to examine this response after longer periods of deprivation, where Peyer's patch germinal centre formation appeared normal (Fig. 6.3(c)).

Finally, the effect of protein deprivation on counts of IELs was not consistent throughout the different periods of restriction. After 2 weeks deprivation, IEL counts in jejunum and ileum were significantly elevated over controls, however, after 6 and 12 weeks, numbers were consistently decreased in all gut samples. The disparate effect on IEL

counts after different periods of restriction has also been suggested from the work of Lyscom and Brueton (1983). They observed that counts in protein deprived rats were normal after 1 and 8 weeks, but were decreased relative to controls during the intervening periods. Differences between these findings and reports from other workers of a reduction in IEL counts (Maffei et al., 1980; Chandra, 1979(b)) can probably be accounted for by differences in the methods used to induce deprivation, and by differences in the periods of restriction after which this parameter was examined.

The increase in IEL counts observed after 2 weeks deprivation is an intriguing finding. While it is possible that the increase in IELs parallels the relative increase in long-lived, recirculating T_2 cells known to occur after short term deprivation (Malavé et al., 1980), the majority of reports argue against the interpretation that the IEL population is long-lived (Marsh, 1985). Nevertheless, IELs are known to be a heterogenous population (Ernst, Befus, Bienenstock, 1985), and one report does suggest that within this population, there may be a subset of small, non-dividing recirculating T cells (Guy-Grand, Griscelli, Vasselli, 1978). The proportion of this subset within the total IEL population therefore, may be increased after short term deprivation. Evidence to support the suggestion that protein deprivation can alter the composition of the IEL population is obtained from the demonstration that the development of the $W3/13^-$ MRC $OX8^+$ subset of IELs in rats is delayed by short-term deprivation (Lyscom and Brueton, 1983).

T Lymphocyte function in protein deprived mice

The experiments presented in Chapter 7 examined primarily T lymphocyte function in the model of protein deprivation in use. Several tests were used, and they provided differing conclusions depending on whether function was assessed *in vivo* within the protein deprived host or when the cells were removed and examined either *in vitro* or within a normal, protein sufficient host. Both the T-dependent antibody response and the DTH response were decreased in short term protein deprived mice, suggesting that T cell function was impaired. However, the results from the GvHR assay indicated that these cells could function normally. The importance of the restrictive environment when interpreting the results of *in vivo* studies has been clearly implied (Pocino and Malavé, 1981), and the data presented in Chapter 7 confirm this proposal.

After short term protein deprivation, the antibody response of BDF_1 mice to OVA contained a high proportion of IgM and a low proportion of IgG. This finding is in agreement with previous reports of the response to several types of antigen in protein deprived animals (Price and Bell, 1977(a): Malavé and Layrisse, 1976). The response was immature in phenotype, and this is consistent with the suggestion that protein deprivation can retard, but not prevent, immunological maturation (Watson and Haffer, 1980).

The T-dependent antibody response is the end result of interaction and cooperation between lymphocytes and antigen presenting cells. It is possible that within this process, several factors can contribute to the altered response observed in these mice. Antigen presenting capacity of peritoneal exudate macrophages *in vitro* has been shown to be impaired in protein deprived mice (Rose *et al.*, 1982). This defect is not related to gross differences in rates of uptake and degradation of antigen between normal and protein deprived mice, but may be due to differences in surface Ia expression and production of the lymphocyte activating monokine, IL-1. It is interesting to note that maturation of the immunogenic properties of macrophages is controlled by a short-lived population of cortical thymocytes (Tzehoval, Segal and Feldmann, 1979). Impaired immunogenic function may then result from the selective depletion of this type of thymocyte, observed after short term protein deprivation (Chapter 6).

A defect in the function of T_h cells has been proposed to account for the depressed IgG responses (Malavé and Layrisse, 1976). This can result from inefficient T-B cell cooperation within the protein deprived environment or from a failure of the T_h cell to produce the lymphokines which promote cellular proliferation and differentiation (e.g. BCGF, TRF). With respect to the latter point, although IL-2 production from protein deprived spleen cells stimulated with ConA is normal *in vitro* (Saxena *et al.*, 1984), it does not follow that this is the case *in vivo*. It is unlikely

that B cell function is severely affected, as the antibody response to T-independent antigens is normal or even increased after short term protein deprivation (Price and Bell, 1977(a); Price, 1978).

An additional explanation for the high proportion of IgM is that protein deprivation can selectively impair the T cells governing the IgM → IgG switch during the course of the response (Price and Bell, 1977(a)). The existence of such a switch cell has been confirmed in mouse spleens by Kawanishi and his colleagues in their report on the T cell responsible for the IgM → IgA switch (Kawanishi, Saltzman and Strober, 1983).

Administration of high doses of corticosteroids to animals shortly before immunization can suppress both the cellular and humoral immune response (Gabrielsen and Good, 1967). Furthermore, increased levels of corticosteroids are known to circulate in the blood of rats after short term protein deprivation (Aschkenasy et al., 1966). Therefore, it is possible that an increase in concentration of this hormone is playing a role in the abnormal response of this model. Its exact contribution is not clear, however, as antibodies of all isotypes (including IgM) are reported to be suppressed by corticosteroids in a dose dependent manner (Benner, Van Dongen and Van Oudenaren, 1978).

When BALB/c mice were protein deprived for 3 weeks before immunization, similar levels of IgM and IgG antibodies were produced in response to OVA compared to control mice.

A sharp contrast exists, therefore, between the effect of protein deprivation on both the physical appearance and antibody response of BDF_1 and BALB/c mice. The difference in the quality of the response between the two strains may be related to the fact that protein deprived BALB/c mice can adapt better to the dietary restriction, and provide a more suitable environment for the lymphocyte interaction and proliferation required for antibody production. With hindsight, it might have proved interesting to examine these topics in a number of different protein deprived mouse strains.

The underlying theme of adaptation after prolonged dietary restriction is again evident in the antibody response. When BDF_1 mice were protein deprived for 11 weeks from weaning before immunization, the antibody response was normal compared to protein sufficient control mice. This improvement in response mirrored the improvement in physical appearance, and is likely to be due to the presence of a better environment for immune responses to occur. Although, it has been reported that mice maintained on a restricted diet for longer periods of time produce near normal total antibody titres, these include increased proportions of IgM (Price and Bell, 1977(b)). This is the first time that the antibody response (both IgM and IgG isotypes) has been demonstrated to recover to normal levels after prolonged deprivation.

A similar pattern was observed with the DTH response. Short-term deprivation impaired the response, but after longer periods, it was restored to values similar to those of control mice. While corticosteroid induced suppression of DTH cannot be ruled out, its exact role in this model is again not clear. An additional explanation was sought in the selective inhibition of one of the limbs of the DTH response.

As represented in Figure 7.4, the DTH response to antigen consists of several steps. A fault in any of these steps can prevent the expression of such a response. A passive cell transfer protocol was devised to study if either the initial priming phase or the inflammatory phase of the response was impaired in the protein deprived host. Due to the profound atrophy of the peripheral lymph nodes of 4% protein mice, it proved impossible to transfer primed cells from here to a protein sufficient footpad with antigen to attempt to elicit the response. Therefore, the effect of protein deprivation on the afferent limb of DTH was not determined. Transfer of 24% protein primed cells to 4% protein footpads did not produce a positive response, suggesting that the inflammatory limb was prevented in short term protein deprived mice. Restoration of this limb, and of the response as a whole, may occur as the animal adapted to longer periods of restriction.

Migration of both lymphocytes and non-specific effector cells to the site of antigen challenge is an essential part

of the DTH response. Failure to elicit this response, therefore, may result from impaired cell migration. This possibility is suggested by the work of Bhuyan and Ramalingaswami (1974), who demonstrated a marked delay and deficiency in macrophage mobilization from protein deprived guinea pigs immunized with BCG. Furthermore, cell recruitment to an antigen stimulated node of a deprived mouse has also been shown to be diminished (Bell et al., 1976(a)).

The GvHR experiment provided a means of comparing the immunocompetence of T cells from normal and protein deprived donors. The reaction was assessed by measuring changes in spleen weight and by examining the changes in mucosal structure caused by a local CMI reaction (Mowat and Ferguson, 1981(b)).

Splenomegaly, characteristic of a GvHR occurred when parental lymphocytes from either 4% or 24% protein groups were used as the donor population. In the strain combination of mice used ($H\text{-}2^{b} \longrightarrow H\text{-}2^{b/d}$), the splenomegaly is almost entirely due to proliferation within the host organ of donor T cells (Ishikawa, Kubota, Wilkinson and Saito, 1982). Furthermore, lymphocytes bearing the $W3/25^{+}$ marker are reported to be the cause of the GvH reactivity (Kimura, Pickard and Wilson, 1984) and this marker is associated with T_h cell activity in rats (Brideau, Carter, McMaster, Mason and Williams, 1980). Therefore, by recognizing and proliferating in response to allogeneic determinants, T_h

cells from protein deprived mice appeared to function normally within protein sufficient F_1 hosts.

This suggestion is supported by the evidence from the mucosal changes in these mice. Both normal and protein deprived lymphocytes were able to induce a limited CMI response in F_1 gut, a reaction attributed to a population of Ly 1^+ DTH effector cells (Mowat, Borland and Parrott, 1986). As help for an antibody response, and the DTH response may be recognized as being different functions of the same T cell (Milon, Marchal, Seman, Truffa-Bachi and Zilberfarb 1983), this implies that the $T_h/_{DTH}$ cell was not irreversibly damaged by protein deprivation.

An increase in the relative number of IELs is taken as one indication of a local CMI reaction in mouse gut (Mowat and Ferguson, 1981(a)). It was interesting to note, however, that in F_1 mice which received protein deprived parental cells, no increase in IEL numbers was observed, despite the alteration in crypt depth, supporting the presence of a CMI reaction. The majority of lymphocytes infiltrating the epithelium of irradiated mice undergoing a GvHR are known to be of donor parental origin (Guy-Grand, et al., 1978). While the origin of the increase in IELs in unirradiated GvHR mice is unknown, a failure to observe this increase might reflect the inability of protein deprived parental lymphocytes to localize and remain within the recipient epithelium.

In summary, the restrictive environment of the protein deprived host can depress normal expression of T cell function.

This is probably accomplished by impairing the important processes of cell migration, cooperation and proliferation. However, when removed from this environment, these cells can be demonstrated to function normally.

The effect of protein deprivation on orally-induced tolerance

A major part of work in this thesis concerned the effect of protein deprivation on the tolerance for systemic antibody and DTH responses which can be induced by feeding. The results demonstrate that protein deprivation had a disparate effect on the humoral and cell mediated limbs of tolerance. While tolerance for antibody responses was enhanced, the degree of suppression of DTH responses following a single feed of antigen, was substantially decreased. These results confirm the hypothesis arising from the experiments of Mowat and his colleagues (Mowat et al., 1982) that separate control mechanisms exist for these two limbs of oral tolerance.

The mechanisms surrounding the induction and maintenance of orally induced tolerance for antibody responses are poorly defined. Therefore, interpretation of these results in terms of mechanisms, is speculative. Two other reports however, have also noticed the phenomenon of enhanced tolerance for antibody responses in protein deprived mice: one dealing with oral tolerance, the second dealing with parenterally induced tolerance. In the first report, Swarbrick (1979) demonstrated that 4% protein mice show

enhanced antibody tolerance to OVA after the antigen has been first encountered orally. The other report is cited in a paper on the effects of protein deprivation on antibody responses to SRBC and B.abortus (Price and Bell, 1977(a)). These authors suggest that tolerance to PVP and pneumococcal polysaccharide is more easily induced in protein deprived mice, than in controls.

The latter report concerns tolerance to T-independent antigens, while the former uses the T-dependent antigen OVA. Enhanced tolerance to both types of antigens, therefore, argues for a common mechanism acting at the level of the B cell, and the ease of tolerance induction in these cells in protein deprived mice may be one reason for this particular effect. The involvement of the B cell in oral tolerance for antibody responses however, remains a point of dispute. While one report has shown that antibody oral tolerance in the response to HGG involves tolerance in both the T cell and B cell compartment (Vives et al., 1980), another conflicting report states that B cells remain fully functional in the tolerant host (Titus and Chiller, 1981). This anomaly may be due to the type of antigen and the tolerizing regime employed. In my model, the question of whether tolerance was induced in B cells of protein deprived mice could be answered by coupling the antigen (OVA) onto LPS, thus removing the need for T cell help in the generation of the response, and enabling an examination of the functional capacity of the tolerant host's B cells (Titus and Chiller, 1981).

In relation to this phenomenon, some additional points arising from this work, are of interest. Analysis of sera using isotype-specific ELISA procedures indicated that both IgM and IgG isotypes were easier to tolerize in protein deprived animals compared to controls. This excludes the possibility that the enhanced tolerance for IgG responses observed in the first experiment in Chapter 8 was due to increased proportions of IgM in the sera of tolerant 4% protein animals. For full expression of the increased tolerance, animals had to be maintained on the 4% protein diet throughout the duration of the experiment. In OVA fed protein deprived animals which were nutritionally re-habilitated at the time of feeding, the degree of tolerance observed was normal (Section D, Chapter 8). In his experiments on enhanced tolerance, Swarbrick (1979) continued OVA fed animals on the deficient diet for 2 weeks after feeding to observe this effect. Tolerance for antibody responses can first be detected four days after the start of oral immunization (Stokes, Newby and Bourne, 1983). In re-habilitated mice, therefore, the mechanisms involved in the induction of antibody tolerance may have recovered and be relatively unaffected by protein deprivation, thus resulting in the normal level of suppression observed. Finally, it proved impossible to tolerize the antibody response of older animals (i.e. 13 weeks old) by feeding, a result also obtained by Lafont *et al.* (1982) using 3 month old DBA/2 mice. A regime of continuous oral immunization in drinking

water or multiple oral doses of antigen may be required to give tolerance in older mice.

By using an adoptive cell transfer protocol, I hoped to examine the effects of protein deprivation on the populations of cells which are responsible for the transfer of tolerance. Specifically, I asked whether the enhanced tolerance for antibody responses was transferable from antigen fed 4% protein mice to naive control mice. Using this system, however, I was unable to transfer suppression of antibody responses with spleen cells from fed mice.

Transfer of tolerance for antibody responses has previously been accomplished using Peyer's patch, mesenteric lymph node and spleen cells from antigen fed rats and mice (Mattingly and Waksman 1978; Richman et al., 1978; Hanson and Miller, 1982). In these cases, antibody responses have been assayed by measuring the PFC response in the spleens of recipient animals. Measurement of serum antibody responses in cell recipients however, indicates that this type of response is not suppressed, even in recipients where the splenic PFC response is (Hanson and Miller, 1982). The effect of the suppressor cells, therefore, is dependent upon the type of assay used to assess the response. With regard to this point, other workers have also failed to transfer tolerance for serum antibody responses using spleen cells from antigen fed donors (Mowat, personal communication).

A possible reason for the inability to transfer tolerance for serum antibody responses lies in the route of transfer of

cells versus the site of the initial immunization. After i.p. transfer, the donor cells may preferentially localize in the spleen, thus providing a means for the greater sensitivity of the splenic PFC response for suppression (Hanson and Miller, 1982). Footpad immunization with antigen would result in major sites of antibody production in the draining popliteal and inguinal lymph nodes. It is reasonable to assume, therefore, that insufficient transferred suppressor cells are concentrated here to inhibit the response. It should theoretically be possible, therefore, to transfer suppression of serum antibody responses by immunizing animals via a different route and/or by giving larger numbers of suppressor cells.

The work of Mattingly and Waksman (1978) also provides a possible explanation for the enhanced oral tolerance in protein deprived mice. The orally induced T suppressor cell in their experiments has the characteristics of a T_2 cell, in that its activity is eliminated following *in vivo* treatment with anti-lymphocyte antiserum, but not by adult thymectomy. Enhanced tolerance for antibody responses may then arise as a result of the relative increase in this type of T cell previously reported to occur in protein deprived mice (Malavé *et al.*, 1980). However, the orally induced T_2 suppressor cell is important in tolerance to SRBC, whereas the phenomenon of enhanced tolerance, in this work, was observed for the antigen OVA. Therefore, in view of recent data indicating that the control mechanisms for oral tolerance

to particulate and soluble antigens may be fundamentally different (Thomas and Mowat, personal communication), the above proposal may not be a valid one.

An intriguing possibility is that increased uptake of antigen through a protein deprived intestine could contribute to the phenomenon of enhanced tolerance by inducing a high-zone tolerance effect similar to that first described by Mitchison (1964). This theory was prompted by two reports of increased uptake of antigen through protein deprived gut (Worthington and Syrotuck, 1976; Rothman *et al.*, 1982(a)). The relevant experiments, however (Chapter 9), revealed that there was no difference in uptake of OVA between protein deprived and normal mice which had been maintained on the diets for 2, 6 or 12 weeks from weaning. A similar result was obtained by Swarbrick (1979), who reported no increase in passage of antigen from the gut lumen to the bloodstream in his model of protein deprivation. Differences between the former reports and these studies could reasonably have resulted from fundamental differences in the types of assay used to assess uptake. Nevertheless, these studies demonstrated that this function of the intestine, in this model, was normal, despite the differences which exist in mucosal structure between protein deprived and normal animals.

Allied to the above suggestion, is the possibility that protein deprivation could alter the form of antigen which is adsorbed through the gut. It has previously been shown that fragments of BSA generated by *in vitro* pepsin

digestion of the protein can induce suppression of antibody responses when injected *in vivo* (Ferguson, Peters, Reed, Pesce and Michael, 1983). Although intestinal processing of antigen does not normally result in antigen fragments in the serum which can suppress antibody responses (Strobel *et al.*, 1983), it was thought that protein deprivation could alter this processing event such that tolerogenic fragments for antibody responses, in addition to those for DTH responses, could be present in the circulation. In the event, however, transfer of serum from OVA fed 4% protein animals suppressed subsequent DTH but not antibody responses in recipient animals. Intestinal processing of antigen, therefore, was normal in protein deprived mice, and enhanced tolerance for antibody responses did not result from a difference in either the quality or the quantity of antigen adsorbed through a protein deprived gut.

Finally, the role of antibody affinity in the appearance of increased antibody tolerance remains to be established. Protein deprivation is known to induce production of lower affinity antibody in mice which are normally high affinity producers (Reinhardt and Steward, 1979). Enhanced tolerance in protein deprived mice may only reflect the presence of increased amounts of low affinity antibody, which do not bind to antigen on the solid phase under the conditions in which the ELISA is performed. This theory, however, does not explain the presence of tolerance in long term protein deprived mice, where SAL fed deprived and control mice

produced similar titres of antibody, and tolerance was not present in OVA fed protein sufficient mice.

The reasons behind the enhanced degree of tolerance for systemic antibody responses remain unclear, and await a better understanding of the mechanisms involved in the induction and maintenance of oral tolerance. In view of the increase in serum cortisol concentrations in protein deprived animals however (Aschkenasy et al., 1966; McFarlane and Hamid, 1973), future experiments will examine the effect of increased levels of this hormone on oral tolerance for antibody responses.

The situation regarding mechanisms of oral tolerance induction for DTH responses, by comparison, is clearer, and therefore permits a realistic attempt at interpretation of the results. I was unable to examine the effects of short term protein deprivation (i.e. 2 weeks before feeding OVA) on DTH oral tolerance, as no positive response in SAL fed control mice could be elicited. After longer periods, however, (i.e. 10 weeks), a positive response in 4% protein mice was restored to normal values. In these mice, feeding antigen before immunization resulted in subsequent suppression of the DTH response, but the degree of tolerance induced was not as profound as was observed in age-matched 24% protein mice. This partial abrogation of suppression was confirmed in short term protein deprived mice which had been changed to the 24% protein diet immediately after they had received antigen orally. In this case, the nutritionally

rehabilitated mice exhibited normal DTH responses, and a decrease in the degree of suppression of DTH observed after OVA feeding. Curiously, this effect of short term protein restriction was not found when deprivation was initiated at maturity instead of weaning. Finally, protein deprivation and CY had the same effect on tolerance for DTH responses, although it was noticeable that protein deprivation had an additional effect on tolerance for antibody responses as has been described previously.

The role of the orally induced T_s cell in the control of DTH oral tolerance is well established (Mattingly and Waksman, 1978; Miller and Hanson, 1979; Mowat et al., 1982; Mowat, 1985). Taken in total, the data from the experiments in Chapter 8 suggest that protein deprivation can selectively deplete a population of T_s cells responsible for the control of oral tolerance. This hypothesis is also prompted by reports which propose that these cells may be deficient in protein deprived hosts (Price and Turner, 1979; Pocino and Malavé, 1981; Saxena et al., 1984; Koster and Pierce, 1985).

Further confirmation of this suggestion was obtained from the results of a cell transfer experiment (Chapter 9). Adoptive transfer of DTH suppression from antigen fed mice is mediated by a population of T cells (Hanson and Miller, 1979; Mowat, 1985). This population, however, was absent from protein deprived animals, as transfer of spleen cells from OVA fed deprived mice to naive recipients before

immunization did not result in suppression of the subsequent DTH response. Furthermore, the serum transfer experiment demonstrated that both protein deprived and normal intestine were capable of generating a serum tolerogen for DTH responses. This effectively excluded abnormal intestinal processing of antigen by a protein deprived gut as a contributing factor in impaired DTH oral tolerance. Moreover, it was also unlikely that this effect occurred as a result of enhanced antigen presenting cell activity within these mice (Strobel et al., 1985), as the immunogenic function of peritoneal macrophages taken from protein deprived mice is reported to be impaired in vitro (Rose et al., 1982).

In the light of these findings, it might have proved interesting to examine tolerance for DTH at another level of dietary restriction e.g. 8% protein. Malavé et al. (1983) demonstrated that the DTH response to SRBC is normal in 8% protein maintained animals. Furthermore, while large numbers of SRBC suppress the response in control mice, this fails to inhibit the response in deprived mice, a fact which the authors attribute to decreased suppressor cell activity after protein deprivation. This level of restriction might have permitted direct examination of the effect of short term protein restriction on oral tolerance for DTH, without the need for nutritional rehabilitation of deprived mice.

It is clear, however, that protein deprivation did not entirely prevent the induction of oral tolerance for DTH responses. This situation was most apparent in the nutritionally rehabilitated OVA fed group (Section D, Chapter 8), where the response remained 44% suppressed compared to control animals, despite it being significantly increased over the response of the tolerant 24% protein group. A similar effect has been observed in the experiments of Mowat et al. (1982), where DTH oral tolerance in mice fed 25 mg OVA is only partially susceptible to CY pretreatment. These authors have argued that other mechanisms of tolerance could be operating in their model e.g. direct anergy of T_h cells, and it is possible that this additional mechanism is also present in OVA fed protein deprived mice.

In summary, protein deprivation has been demonstrated to impair the induction of oral tolerance for DTH responses. It is most likely that this occurs through the selective depletion of a population of short lived T_s cells in deprived mice. These cells are responsible for the fine control of tolerance, although other mechanisms appear to remain functional in these animals to maintain a significant level of suppression of the DTH response.

Local CMI reactions in protein deprived intestine

It has been suggested that a local intestinal suppressor cell system may protect the gut against damaging CMI reactions

to fed antigen (Mowat, 1984). When this system of T_s cells is impaired, however, it is possible to induce an intestinal CMI reaction by feeding antigen, and this is associated with partial abrogation of oral tolerance for systemic DTH responses (Mowat and Ferguson, 1981(a); Mowat et al., 1982).

The results from experiments in Chapter 8 demonstrated that protein deprivation can restrict the induction of oral tolerance for DTH responses. It was predicted, therefore, that protein deprived mice would be susceptible to damaging mucosal CMI reactions induced by feeding antigen. The experiments in Chapter 10 examined this possibility, and also investigated the effector phase of mucosal CMI in protein deprived mice by following the intestinal phase of the GvHR. From these studies, it was apparent that neither a mild GvHR nor continuous oral challenge with antigen in these mice produced the intestinal changes associated with a CMI reaction.

The GvHR experiment provided some additional points of interest. First, in 24% protein mice with GvHR, the maximal increases in IELs and in crypt depth which characterize intestinal GvHR occurred at day 8, despite the fact that the spleen index was greater at day 14 than day 8. In contrast, in neonatal (CBA x BALB/c) F_1 mice undergoing a GvHR, the changes in mucosal architecture parallel the proliferative responses in the spleen (Mowat and Ferguson, 1982), but this has not been studied in adult F_1 mice. Additionally, differences in the strain combinations used

in the two studies (i.e. H-2^b ➡ H-$2^{b/d}$ vs. H-2^k ➡ H-$2^{k/d}$) may have given rise to these anomalies.

Protein malnourished mice with GvHR had significantly larger spleens than control mice on day 14, and assuming that spleen weights in controls would be the same on day 8 as was found on day 14, splenomegaly was probably also present on day 8 in GvHR mice. However, at neither time did this group demonstrate any of the gut changes associated with a CMI reaction. As both the splenomegaly and the mucosal CMI occur as a result of T cell recognition of allogeneic I-A encoded determinants on host cells (Klein, 1977; Mowat, Borland and Parrott, 1986), the lack of mucosal CMI in the protein deprived group was probably not due to an intrinsic fault in the T_{DTH} cells themselves. Rather, I would suggest that the donor effector cells were unable to migrate to the host intestine, and that proliferation of these cells was restricted in the protein deprived environment. Furthermore, recruitment of non-specific effector cells to this site would also be diminished.

The above conclusion is supported by the fact that protein deprived mice did not develop mucosal DTH responses after feeding and continuous oral challenge with OVA, despite their impaired tolerance for DTH and reduced number of T_s cells. This is as yet the only occasion on which impaired oral tolerance for systemic DTH has not predisposed the host to the development of damaging CMI reactions at the gut mucosa. In contrast, 24% protein mice pretreated with

100 mg/kg CY to remove T_s cell activity, did develop mucosal CMI on feeding and challenge with OVA. Interestingly, the increase in crypt depth was not associated with an increased IEL count in this case, but the reason for this is unknown.

The inability to induce a mucosal DTH response in short term protein deprived mice was paralleled by the deficient systemic DTH response described previously (Chapter 7). As systemic DTH responses recovered after longer periods of protein deprivation, it would be interesting to examine whether long term protein deprived animals could also develop a mucosal CMI response after oral challenge with OVA.

These results obtained in protein deprived mice may have important implications for understanding the enteropathy found in humans with malnutrition. Villous atrophy, crypt hyperplasia and lymphocytic infiltration of the lamina propria and intestinal epithelium have been noted in patients with malnutrition, most notably kwashiorkor (Brunser et al., 1965; Tandon et al., 1968). Similarities between the appearance of the small intestine in malnutrition and in coeliac disease have prompted some workers to suggest that these intestinal disorders may arise through a common pathogenic mechanism (Brunser et al., 1965). However, it has been established that the enteropathy associated with coeliac disease may be due to a local CMI reaction (Mowat, 1984). Therefore, my findings that a mucosal CMI reaction cannot be induced in protein deprived mice suggests that the intestinal damage in humans with malnutrition is not analagous to that found in coeliac disease.

Concluding remarks

In the work described in this thesis, I have examined the effects of protein deprivation on the activity and regulation of the immune system in general, but have paid particular attention to immunological events within the intestine. The model of protein deprivation was not intended to be an accurate indicator of the clinical condition of kwashiorkor, but was designed to investigate the ability to manipulate immune function through changes in the diet. In this way, these studies may help to underline how dietary protein deficiency can contribute to the abnormal immune responsiveness found in clinical malnutrition.

There is a large potential for further work using this type of model. One particular area which warrants investigation is the effect of nutritional rehabilitation on the recovery of immune responsiveness, as it has been demonstrated that reintroduction of a protein sufficient diet two days before immunization depresses the primary antibody response to tetanus toxoid (Price and Bell, 1976). A further area of potential for this model of protein deficiency would be to extend our knowledge about the important relationship between protein malnutrition and increased susceptibility to parasitic infections.

Finally, in contrast to these deleterious effects, it should be noted that protein deprivation may also have beneficial effects under certain circumstances. For example, moderate protein malnutrition retards the proliferation of

L 1210 mouse leukaemia cells in BALB/c mice (Petro and Watson, 1982). This increased resistance against leukaemia cell growth is correlated with increased responsiveness of spleen cells to PHA but not with altered development of splenic lymphocyte mediated cytotoxicity. In addition, protein deficiency from weaning can delay the early thymic involution which is characteristic of autoimmune NZB mice (Fernandes, Yunis and Good, 1976(a)), while restriction of both protein and calories delays the onset and depresses the severity of the disease in (NZB x NZW) F_1 hybrid mice (Fernandes, Friend, Yunis and Good, 1978). Furthermore, protein calorie restriction also doubles the lifespan of these mice (Fernandes et al., 1976(b)). These studies, together with the work presented here on the prevention of the mucosal CMI response, demonstrate a potentially beneficial effect to the host of protein deprivation. Nevertheless, it remains to be seen whether carefully controlled periods of nutritional deprivation could be used to alleviate or abrogate potentially harmful immune responses in a clinical situation.

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